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(57) Abstract

This invention relates to cleaved dimers of Mullerian inhibiting substance-like polypeptides. More particularly, this invention relates to such dimers, methods of producing them and methods of using them in the treatment of cancer and tumors, especially those of the female genital tract. The dimers of this invention are also useful in compositions and methods for contraception.

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CLEAVED DIMERS OF MULLERIAN INHIBITING SUBSTANCE-LIKE POLYPEPTIDES

TECHNICAL FIELD OF INVENTION

This invention relates to cleaved dimers of Mullerian inhibiting substance-like polypeptides. More particularly, this invention relates to such dimers, methods of producing them and methods of using them in the treatment of cancer and tumors, especially those of the female genital tract. The dimers of this invention are also useful in compositions and methods for contraception.

BACKGROUND OF THE INVENTION

Mullerian Inhibiting Substance (MIS) is a glycoprotein produced by the Sertoli cells of the embryonic testis. It is a non-steroidal factor that causes regression of the Mullerian duct, the anlagen of the internal female reproductive tract [Jost, Rec. Prog. Horm. Res., 8, 379-418 (1953)]. MIS, in addition to its important role in development, has been shown to be cytotoxic to the human ovarian tumor cell line HOC-21, both in vitro and in vivo (in a nude mouse model) [Donahoe et al., Science, 205, 913-15 (1979); Fuller et al., J. Clin. Endocrinol. Metab., 54, 1051-55 (1982); Donahue et al., Ann. Surg., 194, 472-80 (1981)].

Both human MIS and bovine MIS have been cloned and expressed in various bacterial and animal host cells using both genomic and cDNA sequences. The products of such recombinant transformed cells, as well as those of Sertoli cells, are 70K polypeptides which dimerize to form 140K disulfide-linked dimers. The purified dimers from Sertoli cells or recombinant cells (e.g., CHO cells transfected with an MIS gene) are active in vitro in causing regression of the rat Mullerian duct in a standard organ culture assay [Cate et al., Cell, 45, pp. 685-98 (1986)].

SUMMARY OF THE INVENTION

The present invention relates to dimers of MIS-like polypeptides (bovine, human or other mammal) which may be processed to produce a C-terminal dimer and a N-terminal dimer. These dimers may remain non-covalently associated with each other and are active in a standard organ culture assay for biological activity of an MIS protein [Cate et al., supra]. The associated dimers, which may also be separated from each other by boiling, acidification or treatment with a detergent such as deoxycholate, are useful separately or in combination in the treatment of cancer, especially cancer of the female genital tract. The dimers of this invention are also useful in compositions and methods for contraception.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic description of one process for producing N-terminal and C-terminal dimers of this invention from a dimer of a human MIS.

Figure 2 depicts the genomic DNA of a human MIS. It also depicts the amino acid sequence of immature and mature human MIS -- those sequences are

interrupted by four introns at the DNA level. In this figure, the amino acids are represented by single letter codes as follows:

Phe: F	Leu: L	Ile: I	Met: M
Val: V	Ser: S	Pro: P	Thr: T
Ala: A	Tyr: Y	His: H	Gln: Q
Asn: N	Lys: K	Asp: D	Glu: E
Cys: C	Trp: W	Arg: R	Gly: G

Figure 3 depicts the DNA and amino acid sequences of immature and mature bovine MIS. It is a genomic/cDNA composite sequence.

Figure 4 is a SDS-PAGE of plasmin digested MIS which shows that plasmin cleavage of MIS produces an N-terminal (110 kDa) and C-terminal (25 kDa) dimer.

Figure 5 is a SDS-PAGE which shows that deoxycholate dissociates the non-covalent complex between the N- and C-terminal dimers.

Figure 6 is a schematic outline of the construction of plasmid pD1.

Figures 7A-7D are schematic outlines of the construction of plasmid pJ103.

Figure 8 depicts the nucleotide sequence of oligomers MIS100, MIS103, MIS104, MIS105, and MIS106.

Figure 9 is a SDS-PAGE analysis of purified mutant 103 produced by L9C16 CHO cells. Wild type MIS or mutant 103 were analyzed under reducing and non-reducing conditions, before and after digestion with plasmin.

Figure 10 is a schematic outline of the construction of plasmid pJ100.

Figure 11 is a SDS-PAGE analysis of purified N-terminal dimer produced by L7118 CHO cells.

Figure 12 depicts regression of the Mullerian duct by a combination of the N-and C-terminal dimers.

DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described may be more fully understood, the following detailed description is set forth.

In the description, the following terms are employed:

cDNA clone--A clone containing a DNA insert that was synthesized from mRNA and which does not contain introns. The vector can be a plasmid or a phage.

Genomic clone--A clone containing a DNA insert which is a fragment of a genome (i.e., isolated from total cellular DNA). It can contain introns which interrupt the protein coding region of the gene. The vector can be a plasmid, a phage or a cosmid.

Exon--Portions of the gene which after transcription are maintained in the mRNA following splicing of the precursor RNA.

Intron--Portions of the gene which are spliced out after transcription.

Recombinant DNA Molecule or Hybrid DNA--A molecule consisting of segments of DNA from different genomes which have been joined end-to-end outside of living cells and able to be maintained in living cells.

Expression Control Sequence--A sequence of nucleotides that controls and regulates expression of genes when operatively linked to those genes. They include the lac system, the β-lactamase system, the trp system, the tac and trc systems, the major operator and promoter regions of phage λ, the control region of fd coat protein, the early and late promoters of SV40, promoters derived from polyoma virus and adenovirus, metallothionine promoters, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase,

e.g., Pho5, the promoters of the yeast α-mating factors, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof. For mammalian cells the gene can be linked to a eukaryotic promoter such as that for the SV40 early region coupled to the gene encoding dihydrofolate reductase and selectively amplified in Chinese hamster ovary cells to produce a cell line containing many copies of actively transcribed eukaryotic genes.

MIS-Like Polypeptide -- A polypeptide displaying a biological or immunological activity of an MIS protein. As used herein, the phrase "biological activity of an MIS protein" shall be understood to mean that the MIS-like polypeptide has a cross section of biological activity which is substantially similar to that of a natural MIS protein (e.g., it is able to stimulate regression of the Mullerian ducts, is cytotoxic to one or more types of ovarian tumor cells, for example, the cell line HOC-21, or inhibits growth of endometrial cancer, and preferably, it both stimulates regression of the Mullerian ducts and is cytotoxic to one or more types of endometrial or ovarian tumor cells). As used herein, the phrase "immunological activity of an MIS protein" shall be understood to mean the ability of an MIS-like polypeptide to cross-react with an antibody which is specific for a natural MIS protein. An example of such an antibody is disclosed in United States patent 4,487,833.

A MIS-like polypeptide may include amino acids in addition to those of a native MIS protein or it may not include all of the amino acids of native MIS protein. For example, it may include an N-terminal methionine. Also, this polypeptide may be a mature protein or an immature protein or a protein derived from an immature protein (for example, a

protein wherein only a portion of the signal sequence has been cleaved). Examples of such polypeptides are derivatives of MIS polypeptides which have been prepared by modification of the MIS amino acid sequence to achieve an improvement in properties, e.g., greater storage stability or increased half-life in vivo. As used herein, the phrase "MIS-like polypeptides derived therefrom" shall be understood to mean not only a MIS-polypeptide (e.g., bovine MIS or human MIS) but also various related polypeptides of the types described in this paragraph.

The N-terminal and C-terminal dimers of MIS-like polypeptides of this invention are characterized in that they are produced from a dimer of an MIS-like polypeptide by cleavage between Arg-Ser in the sequence corresponding to RAQRSAGAT of human or bovine MIS (see Figure 1). For example, in the human MIS of Figure 2, that Arg-Ser cleavage site occurs between Arg_{427} and Ser_{428} of mature human MIS. In bovine MIS of Figure 3, the Arg-Ser cleavage site occurs between Arg_{442} and Ser_{443} of mature bovine MIS. In other MIS-like polypeptides, the Arg-Ser cleavage site may be easily located by aligning the sequences with those of mature bovine or human MIS for maximum homology using conventional techniques known in the art.

Thus, two examples of N-terminal dimers of this invention are those comprising disulfide-linked monomers having the following amino acid sequences:

(a) REEVFSTSALPREQATGSGALIFQQAWDWPLSSLWLP
GSPLDPLCLVTLHGSGNGSRAPLRVVGVLSSYEQAFLEAVRRTHWGL
SDLTTFAVCPAGNGQPVLPHLQRLQAWLGEPGGRWLVVLHLEEVTWE
PTPLLRFQEPPPGGASPPELALLVVYPGPGLEVTVTGAGLPGTQSLC
LTADSDFLALVVDHPEGAWRRPGLALTLRRRGNGALLSTAQLQALLF
GADSRCFTRKTPALLLLLPARSSAPMPAHGRLDLVPFPQPRASPEPE
EAPPSADPFLETLTRLVRALAGPPARASPPRLALDPGALAGFPQGQV

NLSDPAALERLLDGEEPLLLLLPPTAATTGVPATPQGPKSPLWAAGL
ARRVAAELQAVAAELRALPGLPPAAPPLLARLLALCPGNPDSPGGPL
RALLLLKALQGLRAEWRGRERSGSARAQR or portions thereof displaying the biological activity of an MIS-like polypeptide

(the monomer of an N-terminal dimer of a bovine MIS); and

(b) LRAEEPAVGTSGLIFREDLDWPPGIPQEPLCLVALGG
DSNGSSSPLRVVGALSAYEQAFLGAVQRARWGPRDLATFGVCNTGDR
QAALPSLRRLGAWLRDPGGQRLVVLHLEEVTWEPTPSLRFQEPPPGG
AGPPELALLVLYPGPGPEVTVTRAGLPGAQSLCPSRDTRYLVLAVDR
PAGAWRGSGLALTLQPRGEDSRLSTARLQALLFGDDHRCFTRMTPAL
LLLPRSEPAPLPAHGQLDTVPFPPPRPSAELEESPPSADPFLETLTR
LVRALRVPPARASAPRLALDPDALAGFPQGLVNLSDPAALERLLDGE
EPLLLLLRPTAATTGDPAPLHDPTSAPWATALARRVAAELQAAAAEL
RSLPGLPPATAPLLARLLALCPGGPGGLGDPLRALLLLKALQGLRVE
WRGRDPRGPGRAQR or portions thereof displaying the biological activity of an MIS-like polypeptide

(the monomer of an N-terminal dimer of a human MIS).

The C-terminal dimers of this invention are characterized by two disulfide-linked monomeric amino acid sequences of about 109 amino acids. They have a molecular weight of about 25K, each amino acid monomer chain having a molecular weight of about 12.5K. The N-terminal dimers of this invention are characterized by two disulfide-linked monomeric amino acid sequences of about 427 amino acids. They have a molecular weight of between about 110 and 115K, each monomeric amino acid chain having a molecular weight of about 57K. The two chains of both the C-terminal dimer and the two chains of the N-terminal dimer may be separated from each other under reducing conditions.

Two examples of C-terminal dimers of this invention are those comprising disulfide-linked monomers having the following amino acid sequences:

(a) SAGAAAADGPCALRELSVDLRAERSVLIPET YQANNCQGACGWPQSDRNPRYGNHVVL LLKMQARGATLARPPCCVPTAYTGKLLISL SEERISAHHVPNMVATECGCR or portions thereof displaying the biological activity of an MIS-like polypeptide (the monomer of a C-terminal dimer of a bovine MIS); and

(b) SAGATAADGPCALRELSVDLRAE RSVLIPETYQANNCQGVCGWPQSDRNP RYGNHVVLLLKMQARGAALARPPCCV PTAYAGKLLISLSEERISAHHVPNMVATEC GCR or portions thereof displaying the biological activity of an MIS-like polypeptide (the monomer of a C-terminal dimer of a human MIS).

The cleaved dimers of this invention may be produced in several ways. In one embodiment, an MIS-like polypeptide is isolated and purified from Sertoli cells using processes such as those described in published European patent application 221,761. These dimeric polypeptides having a molecular weight of about 140K, are then treated with plasmin, trypsin, or other proteases including serum proteases, to generate the N-terminal and C-terminal dimers of this invention. Typically, the N-terminal and C-terminal dimers produced by such plasmin or protease-induced Arg-Ser cleavage remain in non-covalent association with each other.

Although compositions of such associated dimers are useful in the various oncological and contraception applications of this invention, the N-terminal and C-terminal dimers may also be separated from each other by boiling, acidification or treatment with a detergent such as deoxycholate and used separately or together in the compositions and methods of this invention.

In a second embodiment of the processes of this invention for producing novel MIS dimers, the dimers are produced from MIS-like polypeptides produced in unicellular hosts transformed with an expression vector carrying a DNA sequence coding for such MIS-like polypeptides. Such transformed hosts are, for example, described in published European patent application 221,761, which application is incorporated by reference herein. Specific examples of such expression vectors were also deposited in a recognized culture collection and described in that application. These include, for example, E.coli JMB3 (p.BG311.bmis) [IVI 10090] and E.coli JA221 (p.BG312.hmis) [IVI 10089]. These hosts carry animal cell expression vectors that are useful in transforming animal cells to produce bovine (bmis) and human (hmis), respectively.

In this embodiment of the present invention, the transformed host is cultured to produce the MIS-like polypeptide. That polypeptide is then correctly folded to form its disulfide bridges and treated as above with plasmin, trypsin, or other protease, to produce the desired dimers. Again, the N-terminal and C-terminal dimers typically remain in non-covalent association after such treatment. However, if desired, they may be separated by boiling, acid treatment or treatment with a detergent such as deoxycholate before they are used in the compositions and methods of this invention.

In a third embodiment of the processes of this invention for production of novel MIS dimers, the dimers may be produced using recombinant DNA technology. For example, a DNA sequence coding for the amino acid sequence of the monomer of either the C-terminal or N-terminal dimers of MIS-like polypeptides, or both, may be employed using appropriate expression vectors and expression control sequences

to transfect conventional unicellular hosts. Those transformed hosts then produce the monomer that characterizes the desired MIS dimer on fermentation. The required disulfide bridges may then be formed either before, during or after isolation of the produced monomer to produce the desired dimer.

Various techniques well known in the art may be used to isolate a DNA sequence coding for the desired monomer. For example, the processes described in published European patent application 221,761 may be employed to isolate a DNA sequence coding for a mature or immature MIS-like polypeptide. The nucleotide and amino acids of two such polypeptides are depicted in Figures 2 and 3 of this application. They are also carried by plasmids pBG311.bmis and pBG312.hmis deposited in connection with the published European patent application 221,761 and whose construction is described therein.

The DNA sequences coding for the monomers that characterize the desired dimer may then be isolated from the larger DNA sequences by any conventional technique known to those of skill in the art. For example, the larger DNA sequence may be cut with a restriction enzyme at a site, originally present or constructed by site directed mutagenesis, at or near to the "Arg-Ser" cleavage between the N-terminal and C-terminal monomers. If the restriction cut is between the codons that code for the Arg-Ser cleavage site between the N-terminal and C-terminal dimers of MIS, the produced DNA is ready for insertion into an expression vector and transformation into a unicellular host for production of the desired monomer. If the restriction site is upstream or downstream of the Arg-Ser cleavage site, the resulting DNA sequence will need to be shortened by digestion, e.g., using Bal 31, or lengthened by a synthetic oligomer to produce the desired DNA sequence.

A wide variety of host/expression vehicle combinations may be employed in expressing these DNA sequences. For example, useful expression vehicles may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV40 and known bacterial plasmids, e.g., plasmids from E.coli including col E1, pCR1, pBR322, pMB9 and their derivatives, wider host range plasmids, e.g., RP4, phage DNAs, e.g., the numerous derivatives of phage λ, e.g., NM 989, and other DNA phages, e.g., M13 and filamentous single-stranded DNA phages and vectors derived from combinations of plasmids and phage DNAs such as plasmids which have been modified to employ phage DNA or other expression control sequences or yeast plasmids such as the 2μ plasmid or derivatives thereof. For animal cell expression, the preferred expression vectors are pBG311 and pBG312 in Chinese hamster ovary (CHO) cells.

Within each specific expression vehicle, various sites may be selected for insertion of the DNA sequences. These sites are usually designated by the restriction endonuclease which cuts them and are well recognized by those of skill in the art. Various methods for inserting DNA sequences into these sites to form recombinant DNA molecules are also well known. These include, for example, dG-dC or dA-dT tailing, direct ligation, synthetic linkers, exonuclease and polymerase-linked repair reactions followed by ligation, or extension of the DNA strand with DNA polymerase and an appropriate single-stranded template followed by ligation. It is, of course, to be understood that an expression vehicle useful in this invention need not have a restriction endonuclease site for insertion of the chosen DNA fragment. Instead, the vehicle could be joined to the fragment by alternative means.

Various expression control sequences may also be chosen to effect the expression of the DNA sequences. These expression control sequences include, for example, the lac system, the β-lactamase system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage λ, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, promoters for mammalian cells such as the SV40 early promoter, adenovirus late promoter and metallothionine promoter, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses and various combinations thereof. In mammalian cells, it is additionally possible to amplify the expression units by linking the gene to that for dihydrofolate reductase and applying a selection to host Chinese hamster ovary cells.

For expression, DNA sequences are operatively-linked to one or more of the above-described expression control sequences in the expression vector. Such operative linking, which may be effected before or after the chosen DNA sequence is inserted into an expression vehicle, enables the expression control sequences to control and promote the expression of the DNA sequence.

The vector or expression vehicle, and in particular the sites chosen therein for insertion of the selected DNA fragment and the expression control sequence employed in this invention, is determined by a variety of factors, e.g., number of sites susceptible to a particular restriction enzyme, size of the protein to be expressed, expression characteristics such as the location of start and stop codons relative to the vector sequences, and other factors

recognized by those of skill in the art. The choice of a vector, expression control sequence, and insertion site for a particular DNA sequence is determined by a balance of these factors, not all selections being equally effective for a given case.

It should also be understood that the DNA sequences coding for the monomers of the dimers of this invention that are inserted at the selected site of an expression vehicle may include nucleotides which are not part of the actual DNA sequence coding for desired monomer. For example, the DNA sequences may be fused in the same reading frame in an expression vector to at least a portion of a DNA sequence coding for at least one eukaryotic or prokaryotic carrier protein or a DNA sequence coding for at least one eukaryotic or prokaryotic signal sequence, or combinations thereof. Such constructions may aid in expression of the desired DNA sequence or improve purification or permit secretion, of the monomer from the host cell. The DNA sequence may alternatively include an ATG start codon, alone or together with other codons, fused directly to the sequence encoding the first amino acid of the desired monomer. Such constructions enable the production of, for example, a methionyl or other peptidyl MIS monomers. This N-terminal methionine or peptide may either then be cleaved intra- or extra-cellularly by a variety of known processes or the monomers with the methionine or peptide attached may be used, uncleaved, in preparing the dimers of this invention.

The expression vector containing the DNA sequence coding for a MIS monomer is employed in accordance with this invention to transform an appropriate host so as to permit that host to express the monomer for which the DNA sequence codes. Such useful expression hosts may include strains of E.coli, such as E.coli C600, E.coli ED8767, E.coli

DH1, E.coli LE392, E.coli HB 101, E.coli X1776, E.coli X2282, E.coli MRCI, E.coli BNN102, E.col JM83, E.coli JA221, and strains of Pseudomonas, Bacillus, and Streptomyces, yeasts and other fungi, animal hosts, such as CHO cells, COS cells or mouse cells, other animal (including human) hosts, plant cells in culture or other hosts.

The selection of an appropriate host is controlled by a number of factors recognized by the art. These include, for example, compatibility with the chosen vector, toxicity of proteins encoded by the hybrid plasmid, susceptibility of the desired protein to proteolytic degradation by host cell enzymes, contamination or binding of the protein to be expressed by host cell proteins difficult to remove during purification, ease of recovery of the desired protein, expression characteristics, bio-safety and cost. A balance of these factors must be struck with the understanding that not all host vector combinations may be equally effective for either the cloning or expression of a particular recombinant DNA molecule.

It should be understood that the monomers (prepared in accordance with this invention in those hosts) may include polypeptides in the form of fused proteins (e.g., linked to a prokaryotic, eukaryotic or combination N-terminal segment to direct excretion, improve stability, improve purification or improve possible cleavage of the N-terminal segment), in the form of a precursor of the monomer (e.g., starting with all or parts of a MIS-like polypeptide signal sequence or other eukaryotic or prokaryotic signal sequences), in the form of the mature monomer, or in the form of an f-met-monomer.

One particularly useful form of a polypeptide in accordance with this invention, or at least a precursor thereof, is a MIS monomer with an easily

cleaved amino acid or series of amino acids attached to the amino terminus. Such construction allows synthesis of the polypeptide in an appropriate host, where a start signal that may not be present in the monomer is needed, and then cleavage in vivo or in vitro of the extra amino acids to produce the desired monomer. Such methods exist in the art. See, e.g., United States patents 4,332,892, 4,338,397, and 4,425,437. The monomers may also be glycosylated, like native MIS protein, unglycosylated, or have a glycosylation pattern different than that of native MIS protein. Such glycosylation will result from the choice of host cell or post-expression treatment chosen for the particular monomer.

The monomers of the invention also include polypeptides that are coded for on expression by DNA sequences characterized by different codons for some or all of the codons of the native MIS for which the monomer codes. These substituted codons may code for amino acids identical to those coded for by the codons replaced but result in higher yield of the polypeptide. Alternatively, one or a combination of codons may be replaced, leading to amino acid replacement or to a longer or shorter monomer. Such replacements or modifications may alter the properties of the monomer in a useful way (e.g., increase the stability, increase the solubility or increase the therapeutic activity).

The MIS dimers of this invention are useful alone or in combination as anti-cancer drugs. For example, such compositions may comprise an anti-cancer effective amount of an MIS dimer of this invention and a pharmaceutically acceptable carrier. Such therapies generally comprise a method of treating patients in a pharmaceutically acceptable manner with those compositions.

Generally, the pharmaceutical compositions of the present invention may be formulated and administered using methods similar to those used for other pharmaceutically important polypeptides (e.g., alpha-interferon). Thus, the polypeptides may be stored in lyophilized form, reconstituted with sterile water just prior to administration, and administered intravenously. Preferably, the pharmaceutical formulation of the present invention will be administered in dosages and modes of administration similar to those that have been used for MIS protein as disclosed in United States patent 4,510,131, the disclosure of which is hereby incorporated by reference.

According to this invention, MIS dimers may be administered to the patient in any pharmaceutically acceptable dosage form, including those which may be administered to a patient intravenously as bolus or by continuous infusion over a period of minutes, hours, days, weeks or months, intramuscularly, subcutaneously, intracutaneously, intra-articularly, intrasynovially, intrathecally, periostally, or by oral, topical, or inhalation routes. MIS dimers may also be administered intratumorally, peritumorally, intralesionally or periolesionally to exert local as well as systemic therapeutic effects.

The most effective mode of administration and dosage regimen of MIS dimers will depend upon the type of disease to be treated, the severity and course of that disease, previous therapy, the patient's health status and response to the MIS dimers and the judgment of the treating physician. MIS dimers may be administered to the patient at one time or over a series of treatments until the desired suppression of cancer of the female genital tract is achieved.

The MIS dimers of this invention are also useful alone or in combination in compositions and methods for contraception. For example, such com-

positions may comprise an effective amount of the MIS dimers of this invention and a pharmaceutically acceptable carrier. An effective amount of the MIS dimers of this invention is one which is sufficient to achieve contraception in an ovulating human or animal.

The most effective mode of administration and dosage regimen of MIS dimers will depend upon the age, weight, physical condition, medical history, sensitivity of the patient and the judgment of the treating physician. The MIS dimers of this invention may be administered to the patient in any pharmaceutically acceptable dosage form, including administration as a bolus over a period of time in discreet and separable dosages.

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only and are not to be construed as limiting the scope of the invention in any manner.

EXAMPLES

Purification Of MIS

We produced human recombinant MIS from CHO cell line 311-2A9B7 (described in published European patent application 221,761) and from CHO cell line L258B9 transfected with the human MIS gene. All of the following examples used human recombinant MIS from CHO cell line L258B9.

We produced cell line L258B9 as described below. 18.9 µg of plasmid pBG311.hmis was linearized with Asp700 and 1.1 µg of pAdD26 was linearized with StuI and introduced into CHO DHFR$^-$ cells using the calcium phosphate protocol as described in S.J. Scahill et al., "Expression and Characterization of the Product of a Human Immune Interferon cDNA Gene In Chinese Hamster Ovary Cells," P.N.A.S., 80,

4654-4658 (1983). The construction of pAdD26 is described in R.J. Kaufman and P.A. Sharp, "Construction of a Modular Dihydrofolate Reductase cDNA Gene: Analysis of Signals Utilized for Efficient Expression," Mol. Cell. Biol., 2, 1304-1319 (1982). Transformants were selected as described below. We selected CHO cell lines expressing the full length form of MIS in a α-minimum essential medium without ribonucleosides and deoxyribonucleosides (α-medium), which was supplemented with 10% dialyzed fetal bovine serum (FBS). A cell line L258 was obtained expressing about 0.5 mg of MIS per liter of culture medium per day. This cell line was plated in 30 nM methotrexate in order to increase the MIS copy number and thereby increase expression. The cell line thus obtained, L258B9, was produced about 3 mg of MIS per liter per day.

We grew the transfected cells at 37°C in a minimum essential medium without ribonucleosides and deoxyribonucleosides, which was supplemented with 10% fetal calf serum. The conditioned medium was clarified by filtration and concentrated 15-fold by ultrafiltration. We purified the MIS from the concentrate by affinity chromatography on a M10.6 immunoaffinity column using Mab 10-6, a monoclonal antibody raised against the recombinant protein. We then eluted MIS from the affinity matrix with 2M NaSCN, 150 mM NaCl, 15 mM sodium phosphate, pH 6.3, essentially as described in J.-Y. Picard and N. Josso, Mol. Cell. Endocrinol., 84, pp. 23-29 (1984). The chaotrope was removed and replaced with MIS storage buffer (10% glucose, 300 mM NaCl, 10 mM HEPES, pH 7.5) in a P-6DG desalting column (Bio-Rad). We aliquoted and stored the final preparation at -70°C.

We also metabolically labeled the MIS with [^{35}S] cysteine by pulse - labelling the transfected CHO cells (L258B9) for 80 minutes with [^{35}S] cysteine

(approximately 600 Ci/mmol) in cysteine-free minimum essential medium containing 500 μCi/ml of the radioactive amino acid and then chasing the label for 20 hours with complete growth medium. The recombinant MIS was purified from the labelling medium by immune precipitation using the M10.6 monoclonal antibody and eluted from the immune complex with nonreducing electrophoresis sample buffer. After releasing the MIS with nonreducing buffer, a portion of the sample was reduced with 2% 2-mercaptoethanol.

On SDS-polyacrylamide gels, the affinity purified protein migrated under reducing conditions with an apparent mass of 70 K. Under non-reducing conditions, the protein migrated with an apparent mass of 140 K. In addition to these dimeric species of MIS, we observed other higher molecular weight disulfide-linked oligomers. These larger forms also were obtained with radioactive MIS that was pulse-labeled with [^{35}S] cysteine and analyzed directly by SDS-PAGE and with preparations that were disrupted with SDS in the presence of 50 mM iodoacetic acid, indicating that the oligomers were not generated by sample preparation. While the organization of disulfide bonds in MIS has not been determined, the presence of the oligomers suggests that one or more cysteines have free sulfhydryl groups. The amino-terminal sequence of the purified protein was Leu-Arg-Ala-Glu-Glu-Pro-Ala-Val-Gly-Thr, which is consistent with the amino terminus of mature MIS.

In addition to 70 K MIS, a fraction of the purified protein exists as a 57 K form. The amount of that form ranges between about 5 and 20% of the total protein. A small fraction with an apparent mass of 12 K was also observed, suggesting that the 57 K and 12 K products may be generated by proteolysis. Under nonreducing conditions, the 12 K

species migrated as a 25 K dimer. We then subjected the 57 and 12 K dimers to protein sequencing.

The sequence of the N-terminal dimer was Leu-Arg-Ala-Glu-Glu-Pro-Ala-Val-Gly-Thr, indicating that it was derived from the amino terminus of MIS, while the sequence of the C-terminal fragment was Ser-Ala-Gly-Ala-Thr-Ala-Ala-Asp-Gly-Pro, indicating that it was derived from near the carboxyl terminus. Thus, 5-20% of the protein in MIS preparations is cleaved at a site 109 amino acids from the carboxyl terminus (Figure 1).

Plasmin Digestion Of Purified MIS

We diluted the purified MIS 1:1 with water and incubated it at room temperature with plasmin (Sigma) at a constant MIS to plasmin ratio of 25:1 (w/w). We incubated MIS samples with the plasmin at room temperature in 5% glucose, 150 mM NaCl, 5 mM HEPES, pH 7.4, for between about 10 to 120 minutes, typically 60 minutes. The proteolysis was stopped by diluting the samples with electrophoresis sample buffer and heating the preparations at 65°C for 10 minutes. We analyzed the cleavage products by SDS-PAGE on 12% polyacrylamide gels using the Laemmli system [U.K. Laemmli, Nature, 227, pp. 680-85 (1970)] and staining with Coomassie Brillant Blue. The reactions for organ culture assays were quenched by adding fetal bovine serum to 10% and for gel filtration by adding acetic acid to 1M. The organ culture assay was carried out as described in P.K. Donahue et al., J. Surg. Res., 23, pp. 141-48 (1977).

We observed that when purified MIS was incubated with plasmin for 1 hour, the protein was cleaved into a 57 K species and a 12 K species. The sizes of the species and subsequent sequencing of the cleavage products revealed that they were the same species which we had previously observed as

minor components in the purified MIS preparations. In the absence of a reducing agent, the 12 K species migrated as a 25 K dimer and the 57 K species migrated as a 110 K dimer.

Within the 2 hour incubation period, only the 57 K and 12 K species were generated. With longer incubation times or with higher plasmin concentrations, an additional cleavage within the 57 K species was observed, generating a 33 K and a 24 K fragment. MIS could also be selectively cleaved with trypsin to generate the 57 and 12 K species, with the reaction being less controlled. With trypsin, both species were susceptible to further digestion.

We then subjected the 57 and 12 K dimers produced by plasmin digestion to protein sequencing. Samples containing either 0.5 nmol of intact protein or 0.25 nmol of the plasmin-derived fragments were subjected to automated Edman degradation in an Applied Biosystems (ABI) 470A gas phase Sequencer in the presence of Polybrene [R.M. Hewich et al., J. Biol Chem., 256, pp. 7990-97 (1981)]. PTH amino acids were analyzed on-line using an ABI 120 PTH analyzer. The amino acid composition of parallel aliquots were determined in a Beckman System 6300 amino acid analyzer. The sequence of the N-terminal dimer was Leu-Arg-Ala-Glu-Glu-Pro-Ala-Val-Gly-Thr, indicating that it was derived from the amino terminus of MIS, while the sequence of the C-terminal fragment was Ser-Ala-Gly-Ala-Thr-Ala-Ala-Asp-Gly-Pro, indicating that it was derived from near the carboxyl terminus.

As previously described, the 57 and 12 K fragments from a preparation of affinity-purified MIS were isolated by preparative SDS-PAGE and subjected to amino-terminal sequencing. The observed sequences were identical to those generated from plasmin-treated MIS. Although the source of the

protease in conditioned medium responsible for the processing is not known, the sequencing results indicate that it and plasmin cleave MIS at the same site.

Purification and Properties of the Carboxyl-Terminal Dimer of MIS

While digestion by plasmin caused the quantitative cleavage of the MIS, the resulting dimers remained associated as a noncovalent complex The dimers could be separated by acidifying the sample with 1M acetic acid, by boiling, or by detergent treatment with deoxycholate. Also, a nonionic or another ionic detergent which dissociates the fragments without a concomitant loss of their biological activity may be used. Specifically, 2 mg plasmin-treated MIS in 1 M acetic acid was subjected to gel filtration on a P-150 column (1.5 x 40 cm) in 1 M acetic acid at a flow rate of 2 ml/h. Fifty 0.5 ml fractions were collected. Samples were tested for absorbance at 280 nm and for composition by SDS-PAGE for gel analysis, aliquots were dried with a Speed Vac Concentrator (Savant) and resuspended in electrophoresis sample buffer.

In 1 M acetic acid, both MIS dimers were soluble and could be separated by gel filtration chromatography in acetic acid. The two dimers were completely resolved. After removal of the acetic acid by lyophilization, we found that the dimers were soluble in 4 mM HCl, as well as in PBS at neutral pH.

After boiling plasmin-cleaved MIS for 10 minutes, the C-terminal dimer remained soluble, while most of the N-terminal dimer precipitated and could be removed by centrifugation.

Alternatively, the two dimers were dissociated with 1% sodium deoxycholate as detailed below.

Concentrations of sodium deoxycholate between 0.5% and 1.5% also dissociated the two dimers.

We then subjected the carboxyl-terminal dimer to amino acid analysis. Table I below shows theoretical and observed amino acid compositions for the dimer and for intact MIS.

TABLE I

	MIS		Dimer	
Amino acid	Theoretical	Actual	Theoretical	Actual
Cysteine				
Aspartic acid	32	32.6	8	8.1
Threonine	25	26.7	4	5.3
Serine	28	23.9	7	6.2
Glutamic acid	47	47.3	10	10.8
Proline	65	63.5	8	9.0
Glycine	49	49.0	8	8.5
Alanine	71	70.8	15	14.6
Valine	28	26.9	8	6.2
Methionine				
Isoleucine	5	5.6	3	2.6
Leucine	87	88.8	11	12.1
Tyrosine	6	7.0	3	2.6
Phenylalanine	9	9.1	0	0.7
Histidine	7	7.0	3	2.4
Lysine	3	4.0	2	2.0
Arginine	51	49.4	9	8.6

As shown in Table I, the theoretical and observed compositions of the dimer show a good correlation. In particular, the presence of 9 arginines (since arginine is the last amino acid in the fragment) supports that no other processing has occurred. For comparison, the theoretical and observed compositions for intact MIS are shown, which also demonstrate a good correlation.

We have shown below the activities of the various species in the organ culture assay (regression of the Mullerian duct). Both full length and plasmin - digested MIS caused regression of the Mullerian duct in the organ culture assay. The specific activity of the MIS was unaffected by the cleavage. The results are displayed in Table II.

TABLE II

MIS Preparation**	Dose Response* 1:20 Dilution	1:40 Dilution	1:50 Dilution
intact MIS	5	5	
Plasmin-digested MIS	5	5	3
MIS + 1M acetic acid	0	0	
digested MIS + 1M acetic acid	2-3	0	
MIS + boiling	3	0-1	
Digested MIS + boiling	0	0	
C-terminal dimer isolated after acid treatment or boiling	0	0	
Digested MIS + deoxycholate			3-4

These assays demonstrate that the plasmin-cleaved dimers when associated have similar activity in the assay to intact MIS. Plasmin digestion of the 140 K dimer of MIS did not alter its activity in the organ culture assay. However, treatment with acid or boiling, although permitting dissociation of the two dimers, appears also to reduce the activity of both the intact MIS and cleaved MIS. Treatment with sodium deoxycholate permitted dissociation of the two fragments, and did not alter the activity of the intact and cleaved MIS.

Deoxycholate Dissociates The Non-Covalent Complex and Does Not Affect Biological Activity

We digested 250 µg of human recombinant MIS (as prepared above in the Example entitled

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* The dose response is measured by grades: 5 = 1:20 dilution; 4-5 = 1:40 dilution; 3 = 1:80 dilution; 2 = 1:160 dilution.

** All MIS concentrations were 330µg/ml, except that the C-terminal dimer isolated after acid treatment or boiling was used at 100µg/ml.

Purification of MIS) with 25 µg of plasmin in a 1 ml reaction containing 5mM HEPES (pH 7.4), 150 mM NaCl, and 5% glucose for 60 min at 25°C. The reaction was stopped by adding soybean trypsin inhibitor to 100 ug/ml and the extent of digestion was analyzed by SDS-PAGE. This showed that the 140 K dimer of MIS had been completely cleaved to generate a 110 K N-terminal dimer and a 25 K C-terminal dimer. It was previously reported that the N-terminal cleavage product was a trimer. This was apparently due to a gel artifact, which can be eliminated by using a modified Laemmli gel system as described below. Figure 4 shows the results of a titration with plasmin as analyzed by SDS-PAGE under reducing and non-reducing conditions (1 µg per lane) on 10% gels using the Laemmli system (Laemmli, U.K., Nature, 237, 680-685 (1970)) with the following modifications. The concentration of Tris-HCl (pH 6.8) in the stacking gel was 125 mM and the concentration of Tris-HCl (pH 8.8) in the gel was 370 mM. Proteins were stained with Coomassie Brilliant Blue. The ratio of MIS to plasmin (weight/weight) was 10 (lane 2), 20 (lane 3), 40 (lane 4), or 80 (lane 5). Lane 1 contains undigested MIS. Under non-reducing conditions and with this gel system, the N-terminus migrates as a dimer.

We bound the non-covalent complex to an immunoaffinity matrix that was prepared according to the following procedure. A mouse monoclonal antibody M10.6 which was raised against human recombinant (obtained from CHO cell line 311-2A9B7) MIS and recognizes an epitope on the N-terminal domain, was purified from mouse ascites by a combination of protein A Sepharose and ion-exchange (Fast Q) chromatography, and coupled to Affigel-10 using the manufacturer's directions. We transferred 250 µl of this matrix containing the M10.6 antibody to a 1.5 ml Eppendorf tube and washed 3 times with

PBS, 2 times with 2 M NaSCN, and three times with PBS (1 ml per wash). We then added the plasmin cleaved MIS (1 ml) to the washed matrix, and rocked the Eppendorf tube for 90 min at 4°C. The matrix was then washed three times with PBS (1 ml per tube), and transferred to a disposable 2 ml glass pipet that had been plugged with siliconized glass wool.

The C-terminal dimer was eluted from the column with 1% sodium deoxycholate (10mM HEPES, pH 8.0, 150mM NaCl) which was applied in 6 fractions of 125 µl. SDS-PAGE showed that only the C-terminal dimer was eluted with this treatment (Figure 5, lane 1). 10 µl of one of the deoxycholate fractions was analyzed in lane 1. We then washed the column with PBS (5 ml) and eluted with 2 M NaSCN applied in 6 fractions of 125 µl. Ten µl of one of the 2 M NaSCN fractions was analyzed in lane 2. These fractions contained both the N-terminal dimer and the C-terminal dimer. The gel conditions were similar to those described for Figure 4, with the following exceptions. The acrylamide percentage of the stacking gel and gel was 7.5% and 12%, respectively. The concentration of Tris-HCl (pH 6.8) in the stacking gel was 62 mM and concentration of Tris-HCl (pH 8.8) in the gel was 400 mM. The concentration of Tris base and glycine in the running buffer were 50 mM and 500 mM, respectively.

To demonstrate that 1% sodium deoxycholate did not affect biological activity, we cleaved MIS with plasmin as described above, except that the MIS concentration was 667 µg/ml. 10 µl of this plasmin cleaved MIS was added to either an Eppendorf tube with 15 µl of MIS buffer (10 mM HEPES, pH 7.4, 300 mM NaCl, 10% glucose) or to a tube with 12.5 µl of MIS buffer and 2.5 µl of 10% sodium deoxycholate. The two tubes were incubated on ice for 5 min. We then added 88 µl of cold MIS buffer to both tubes.

The tubes were maintained on ice for 3 hours, after which 1 ml of organ culture medium was added. The diluted samples were sterile filtered, and introduced in duplicate into the organ culture assay (Donahoe et al. J. Surg. Res., 23, 141-148 (1977)) to measure biological activity. The sample not treated with deoxycholate produced grade 3 regression of the Mullerian duct in both assays, while the sample treated with deoxycholate produced grade 3 and grade 3-4 regression in the two assays. Thus, treatment of plasmin-cleaved MIS with deoxycholate did not affect biological activity.

Construction Of pD1

We constructed pD1 via a four way ligation shown in Figure 6 with the following four fragments: 1) 271 bp StuI-MstII fragment from pGAP1.6; 2) 323 bp MstII - XhoI fragment from pMIS D/F; 3) 1299 bp XhoI - StuI fragment from pBG312.hmis; and 4) the 6251 bp StuI fragment from pBG312.hmis.

We inserted the 4.5 kb AflII fragment from chmis33 into the animal cell expression vectors pBG311 and pBG312 described by Cate et al. (Cell, 45, 685-698 (1986)), to produce pBG311.hmis and pBG312.hmis, respectively. pBG311 uses the SV40 early promoter, while pBG312 uses the adenovirus-2 major late promoter to drive expression.

We generated plasmid pGAP1.6 which is missing the first intron of the human MIS gene through gapped mutagenesis. The 1600 bp PvuII fragment from chmis 33 was subcloned into the SmaI site of pUC18 to generate pUC18.PV2. This plasmid was linearized with SspI, denatured and then annealed to denatured pUC18.PV2 digested with StuI and MstII. This permitted the formation of hybrid duplexes between the SspI digested and the StuI and MstII digested pUC18.PV2. We then annealed oligomer MIS-42 con-

taining sequence from the 3' end of exon 1 of the human MIS gene and the 5' end of exon 2 of the human MIS gene (i.e., missing the first intron) to the hybrid duplexes.

The sequence of MIS-42 is:

5' TGTTGGCTCC CAGGTCACTT CCTCCAGGTG TAGG 3'.

We used DNA polymerase I - large fragment to synthesize the second strand. We then transformed E. coli and screened colonies with the ^{32}P-labeled oligomer. We identified a positive clone, pGAP1.6, and sequenced it to verify that the first intron was deleted. We isolated the 271 bp StuI-MstII fragment for the four way ligation (Fig. 6).

The construction of pMIS D/F in which introns 2, 3, and 4 are deleted involved two steps. In the first step, we isolated a lambda clone λMIS21 from a λgt10 cDNA library made from RNA that was isolated from COS cells transfected with pBG312.hmis (see above). We sequenced the insert of this clone and determined that introns 3 and 4 of the human MIS gene were missing. In the second step, we isolated the 269 bp AvaI - XhoI fragment of MIS21 that spans from exon 3 to the 5' end of exon 5 and ligated it to a linker and the XhoI - HindIII fragment of vector pcHSA35 (described below). The linker was made by synthesizing two oligomers (MIS-56 and MIS-57) of 63 nucleotides containing the DNA sequence from the MstII site in exon 2 to the AvaI site in exon 3, but missing intron 2 of the human MIS gene. In addition, the linker contained DNA sequence encoding a HindIII site at the 5' end (adjacent to the MstII site). The sequence for MIS-56 (top strand) and MIS-57 (bottom strand) is:

5' AGCTTCCTGAGGTCACTGTGACGAGGGCTGGGCTGCCGGGTGCCCAGAGCCTCTGCCCCTC 3'
3' AGGACTCCAGTGACACTGCTCCCGACCCGACGGCCCACGGGTCTCGGAGACGGGGAGGGCT 5'.

The three way ligation produced plasmid pMIS D/F which is missing introns 2, 3, and 4 of the human MIS gene. The 323 bp MstII - XhoI fragment was then isolated for the four way ligation (Fig. 6).

pcHSA35 is a plasmid constructed from plasmid pcHSA36. pcHSA36 was deposited in the culture collection of the American Type Culture Collection in Rockville, Maryland on December 9, 1982 and identified there as HSA-B and assigned ATCC accession number 39253. pcHSA36 was digested with restriction enzyme BstEII to completion, blunt ended with the exonuclease Bal31, followed by digestion with the restriction enzyme BamHI and the sticky ends blunt ended with DNA polymerase I - large fragment. The resulting linear plasmid was circularized by ligation and a plasmid containing a single XhoI site was isolated and designated pcHSA35.

A non-cleavable mutant of MIS is inactive

A mutant protein of MIS was not cleaved with plasmin, and was inactive in the organ culture assay when Arg 427 within the cleavage site was changed to a threonine.

Plasmid pJ103, encoding for a non-cleavable form of human MIS, in which Arg 427 is converted to a threonine, was generated in three steps (Figures 7A, 7B, 7C, 7D). Plasmid pJ103 also contains the adenovirus-2 major late promoter driving transcription of the altered MIS cDNA, and a copy of the mouse dihydrofolate reductase (DHFR) cDNA under the control of the SV40 early promoter. These features permit the expression of the altered form of MIS in Chinese hamster ovary (CHO) cells.

We first inserted oligomers MIS-103, MIS-104, MIS-105, and MIS-106 (Figure 8) into the BamH1 and Sac1 sites of plasmid pUC-MIS98 to generate pUC-MIS103 (Figure 7A). Plasmid pUC-MIS98 contains

the Nar1-Sac1 fragment of plasmid pD1 in the Nar1 and Sac1 sites of vector pUC-18 (commercially available from International Biotechnologies, Inc., catalog No. 33510 (1986-87)). The construction of pD1 is described above. The nucleotide sequence of plasmid pUC-MIS103 contains a mutation, which changes Arg 427 of human MIS to a threonine.

Next, we inserted the Nar1-Sac1 fragment of pUC-MIS103 which contains the mutation into the Nar1 and Sac1 sites of plasmid PTZ-MIS to generate PTZ-MIS103 (Figure 7B). Plasmid PTZ-MIS contains the Hind3-Not1 fragment of pD1 in the Hind3 and EcoR1 sites of vector pTZ-18R. Prior to the ligations, the Not1 site of the fragment and the EcoR1 site of the vector were converted from 5' overhangs to blunt ends using DNA polymerase I - large fragment and all four deoxynucleotide triphosphates.

Finally, we inserted the Bsu36 I-BstE2 fragment of plasmid PTZ-MIS103 containing the mutation into the Bsu36 I and BstE2 sites of plasmid PJOD-10 to generate plasmid pJ103 (Figure 7D).

The vector pJOD-10 (Figure 7C) contains the human MIS cDNA. The MIS cDNA is expressed from the Adenovirus 2 major late promotor ("AdMLP"), along with the SV40 enhancer. Downstream from the MIS poly A addition site and 3'-genomic flanking sequence are SV40 splice and polyadenylation sites. This vector also contains the murine dihydrofolate reductase ("DHFR") cDNA. The DHFR gene is expressed from the SV40 early promoter and is followed by SV40 splice and polyadenylation signals. The DHFR and MIS cDNA are expressed in opposite orientations. Between the two SV40 poly A sites is a transcriptional termination element. This element was synthesized as an oligonucleotide homologous to the human gastrin gene transcriptional termination sequence (see Sato et al., "A Specific DNA Sequence

Controls Termination of Transcription in the Gastrin Gene", Mol. Cell. Biol., 6, pp. 1032-43 (1986)). This terminator element is employed in order to block transcriptional interference between the MIS and DHFR cDNA. The pJOD-10 vector also contains the ampicillin-reistance gene and the ColE1 bacterial origin of replication derived from pBR327, allowing cloning and amplification of this vector in bacteria.

Vector pJOD-10 was constructed (see Figure 7C) from DNA of three origins: (1) vector PD1 (which comprises the human MIS gene); (2) vector pSV2-DHFR (which comprises the murine DHFR gene); and (3) synthetic oligonucleotide homologous to the human gastrin gene transcriptional terminator. The construction of pD1 is described in Cate et al., European patent application 221,761. The construction of vector pSV2-DHFR is described in Subramani et al., "Expression of the Mouse Dihydrofolate Reductase Complementary Deoxyribonucleic Acid in Simian Virus 40 Vectors", Mol. Cell. Biol., 1(9), pp. 854-864 (1981) and is available from the American Type Culture Collection (ATCC 37146).

Two complementary oligonucleotides homologous to the human gastrin transcriptional terminator were synthesized according to standard procedures using an Applied Biosystems 380A DNA Synthesizer. These oligonucleotides were isolated by gel chromatography. The oligonucleotide corresponding to the gastrin gene coding strand is 51 nucleotides long and comprises a sequence homologous to nucleotides +190 to +233 of the human gastrin gene, according to the map coordinates and sequence of Sato et al., supra. The complementary oligonucleotide is 59 nucleotides long. These oligonucleotides were annealed, forming a double stranded DNA molecule ("term") with an ApaI overhang at one end and an XhoI site and EcoRI overhang at the other end:

```
          +190                                              +233
           |                                                 |
5'     CCTTTTTTTTTAATTTTTATTTTATTTTATTTTTGAGATGGAGTCTCGAGG     3'
3' CCGGGGAAAAAAAAATTAAAAATAAAATAAAATAAAAACTCTACCTCAGAGCTCCTTAA 5'.
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Vector pSV2-DHFR was cut with EcoRI and ApaI and the large fragment was gel purified. The double stranded term insert was then ligated into the ApaI/EcoRI pSV2-DHFR fragment, forming vector pDT4. Vector pDT4 was cut with AatII and XhoI and the large fragment was gel purified. Vector pD1 was cut with SalI and AatII and the large fragment was gel purified. The SalI/AatII pD1 large fragment was inserted into the AatII/XhoI pDT4 large fragment, forming pJOD-10.

Plasmid pJ103 was linearized with the restriction endonuclease Pvul and introduced into CHO DHFR$^-$ cells using the calcium phosphate procedure (Scahill, S.J. et al., supra). We selected CHO cell lines expressing the mutant form of MIS in α minimum essential medium without ribonucleosides and deoxyribonucleosides (α-medium), which was supplemented with 10% dialyzed fetal bovine serum (FBS). A cell line (L9C16) expressing in the range of 0.5 mg. of mutant MIS per liter of medium per day was identified using a sandwich ELISA. The cell line was grown in T150 flasks in α-medium containing 5% dialyzed FBS. After the cells were confluent, we replaced the medium every 2-3 days. We then collected 3.3 liters of medium, and clarified by filtration and concentrated to 300 ml using an Amicon concentrator CH2PR with a 30,000 molecular weight cut-off hollow fiber cartridge (H1P30-20). We purified the mutant protein from the concentrate by immunoaffinity chromatography using the M10.6 monoclonal antibody. Mutant MIS was eluted from the affinity matrix with 2 M NaSCN, 150 mM NaCl, 15 mM sodium phosphate, pH 6.3. We removed the chaotrope and replaced it with MIS buffer in a P-6DG desalting column (Bio-Rad).

1.2 mg of the mutant protein was recovered, frozen and stored at -70°C.

SDS-PAGE showed that the mutant MIS is a 140 kDa dimer. When the mutant protein was digested with plasmin using the conditions described above, no cleavage was observed (Figure 9). Using the same conditions as described for Figure 4, wild type MIS was completely cleaved into the N- and C-terminal dimers.

When we assayed the mutant protein in the organ culture assay, no activity was observed, even at concentrations of 30 µg/ml. In contrast, wild type MIS produces grade 5 regression of the Mullerian duct in the range of 2.5 to 5 µg/ml. Thus we observed that the mutant protein is biologically inactive, and we believe that this lack of activity is a consequence of its inability to be cleaved. Cleavage of the MIS 140 K dimer into the N- and C-terminal dimers is, therefore, necessary for biological activity.

Production of the N-terminal dimer

We produced the N-terminal dimer in CHO cells by expressing an altered form of the MIS cDNA in CHO cells.

The plasmid pJ100 encoding the N-terminal domain of human MIS in which the codon for Ser 428 has been converted to a stop codon, was generated in two steps (Figure 10). This plasmid is similar to pJ103 described above and permits the expression of the N-terminal dimer in CHO cells.

First, we introduced a mutation into plasmid pD1, which contains the human MIS cDNA, using site-specific mutagenesis. The construction of pD1 is described in Cate et al., European patent application 221,761. Two forms of pD1 were made, a linear form using NruI and a gapped form using OxaNI

and BstE2. Both of these forms were heat denatured in the presence of an oligomer MIS-100 which contains the mutation (codon for Ser 428 to stop codon), and annealed to produce a heteroduplex with the MIS-100 oligomer bound to the single strand gap in this heteroduplex. DNA polymerase I - large fragment was used to fill in the single strand regions, and the DNA was used to transform E.coli (DH5). A clone was identified containing a plasmid (pD1-100-1) which contained the mutation. This was verified by restriction enzyme mapping and DNA sequencing.

Next, we inserted the OxaN1-BstE2 fragment of plasmid pD1-100-1 containing the mutation into the OxaN1 and BstE2 sites of plasmid pJOD-10 to generate plasmid pJ100.

Plasmid pJ100 was linearized with Aat2 and introduced into CHO DHFR$^-$ cells as described above. A cell line (L7118) expressing the N-terminal dimer of MIS was identified using the sandwich ELISA. Conditioned medium was produced using this cell line and the N-terminal dimer was purified from this conditioned medium as described above.

SDS-PAGE analysis demonstrated that this protein is produced as a dimer, with a slightly larger molecular weight than the N-terminal fragment produced by cleaving full-length MIS with plasmin (Figure 11). Gel conditions are identical to those used for Figure 4. Since endoglycosidase F treatment of the N-terminal dimer produced in cell line L7118 and full length MIS produced in cell line L258B9 which contains about 15% cleaved MIS, converts both N-terminal fragments to the same size, it has been concluded that the increase in size of the N-terminal dimer produced in L7118 is due to more glycosylation.

Production of the the C-terminal fragment

A purification of the C-terminal dimer has already been described above using 1 M acetic acid to break the non-covalent complex formed after cleaving full-length MIS with plasmin as described above under Purification and Properties of the Carboxyl-Terminal Dimer of MIS. Alternatively the C-terminal dimer can be produced using deoxycholate as described above.

Biological activity of the N- and C-terminal dimers

The N- and C-terminal dimers were assayed in the organ culture assay (Donahoe et al., supra.) individually and together. The N-terminal dimer was inactive at a concentration of 40 µg/ml (Figure 12), while the C-terminal dimer (10 µg/ml) prepared using 1 M acetic acid, was also found to be inactive. When the two fragments were assayed together, grade 4 regression of the Mullerian duct was produced (Figure 12). This result indicates that both fragments are necessary to cause regression of the Mullerian duct, and that both fragments may be necessary to inhibit tumor growth.

The C-terminal dimers prepared using 1% deoxycholate can be assayed individually and together with the N-terminal dimers in the organ culture assay to determine which contains biological activity. A positive result observed using the organ culture assay (Donahoe et al., supra) on the N- and C-terminal dimers individually would indicate that each dimer possesses some distinct biological activity. A positive result observed using the organ culture assay (Donahoe et al. supra) on the N- and C-terminal dimers together would indicate that both fragments together are necessary to produce biological activity. (i.e., both fragments in the same assay, not physically together.) They can also

be assayed in primary tumor assays and on cell lines to assess anti-proliferative activity as described in Mosmann, T., "Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays", J. Immunol. Methods 65, 55-63 (1983) and Von Hoff, D.D., B.J. Forseth, M. Huong, J.B. Buchok, B. Lathan, "Improved Plating Efficiencies for Human Tumors Cloned in Capillary Tubes versus Petri Dishes", Cancer Res. 64, 4012-4017 (1986).

While we have hereinbefore described a number of embodiments of this invention, it is apparent that our basic constructions can be altered to provide other embodiments which utilize the processes and compositions of this invention. Therefore, it will be appreciated that the scope of this invenion is to be defined by the claims appended hereto rather than by the specific embodiments which have been presented hereinbefore by way of example.

We claim:

1. N-terminal and C-terminal dimers of an MIS-like polypeptide, said dimers being produced by cleavage between Arg-Ser in the sequence AQRSAG of the MIS-like polypeptide.

2. An N-terminal dimer according to claim 1.

3. The N-terminal dimer according to claim 2, wherein the C-terminal sequence of the monomer of the dimer is AQR.

4. A C-terminal dimer according to claim 1.

5. The C-terminal dimer according to claim 4, wherein the N-terminal sequence of the monomer of the dimer is SAG.

6. The dimers according to claim 1, wherein the N-terminal dimer and C-terminal dimer are associated with each other by a non-covalent interaction.

7. An N-terminal dimer of an MIS-like polypeptide, the momomer of said dimer having an amino acid formula selected from the formulae:

(a) REEVFSTSALPREQATGSGALIFQQAWDWPLSSLWLP
GSPLDPLCLVTLHGSGNGSRAPLRVVGVLSSYEQAFLEAVRRTHWGL
SDLTTFAVCPAGNGQPVLPHLQRLQAWLGEPGGRWLVVLHLEEVTWE
PTPLLRFQEPPPGGASPPELALLVVYPGPGLEVTVTGAGLPGTQSLC
LTADSDFLALVVDHPEGAWRRPGLALTLRRRGNGALLSTAQLQALLF
GADSRCFTRKTPALLLLLPARSSAPMPAHGRLDLVPFFQPRASPEPE
EAPPSADPFLETLTRLVRALAGPPARASPPRLALDPGALAGFPQGQV
NLSDPAALERLLDGEEPLLLLLPPTAATTGVPATPQGPKSPLWAAGL
ARRVAAELQAVAAELRALPGLPPAAPPLLARLLALCPGNPDSPGGPL

RALLLLKALQGLRAEWRGRERSGSARAQR or portions thereof displaying the biological activity of an MIS-like polypeptide ;

and

(b) LRAEEPAVGTSGLIFREDLDWPPGIPQEPLCLVALGG DSNGSSSPLRVVGALSAYEQAFLGAVQRARWGPRDLATFGVCNTGDR QAALPSLRRLGAWLRDPGGQRLVVLHLEEVTWEPTPSLRFQEPPPGG AGPPELALLVLYPGPGPEVTVTRAGLPGAQSLCPSRDTRYLVLAVDR PAGAWRGSGLALTLQPRGEDSRLSTARLQALLFGDDHRCFTRMTPAL LLLPRSEPAPLPAHGQLDTVPFPPPRPSAELEESPPSADPFLETLTR LVRALRVPPARASAPRLALDPDALAGFPQGLVNLSDPAALERLLDGE EPLLLLLRPTAATTGDPAPLHDPTSAPWATALARRVAAELQAAAAAEL RSLPGLPPATAPLLARLLALCPGGPGGLGDPLRALLLLKALQGLRVE WRGRDPRGPGRAQR or portions thereof displaying the biological activity of an MIS-like polypeptide.

8. A C-terminal dimer of an MIS-like polypeptide, the monomer of said dimer having an amino acid formula selected from the formulae:

(a) SAGAAAADGPCALRELSVDLRAERSVLIPET YQANNCQGACGWPQSDRNPRYGNHVVL LLKMQARGATLARPPCCVPTAYTGKLLISL SEERISAHHVPNMVATECGCR or portions thereof displaying the biological activity of an MIS-like polypeptide; and

(b) SAGATAADGPCALRELSVDLRAE RSVLIPETYQANNCQGVCGWPQSDRNP RYGNHVVLLLKMQARGAALARPPCCV PTAYAGKLLISLSEERISAHHVPNMVATEC GCR or portions thereof displaying the biological activity of an MIS-like polypeptide.

9. A pharmaceutically acceptable composition for treating susceptible cancers, said composition comprising an anti-cancer effective amount of at least one of the dimers according to any one of claims 1 to 8.

10. The composition according to claim 9, wherein at least one N-terminal dimer and one C-terminal dimer is present.

11. The composition according to claim 10, wherein the N-terminal dimer and C-terminal dimer are associated by noncovalent interaction.

12. A method of treating susceptible cancers, said method comprising administering to a patient an anti-cancer effective amount of a composition according to any one of claims 9-11.

13. A pharmaceutically acceptable composition for contraception, said composition comprising a contraceptive effective amount of at least one dimer according to any one of claims 1 to 8.

14. The composition according to claim 13, wherein at least one N-terminal dimer and at least on C-terminal dimer is present.

15. The composition according to claim 14, wherein the N-terminal dimer and the C-terminal dimer are associated by noncovalent interaction.

16. A process for producing at least one of an N-terminal dimer or C-terminal dimer of an MIS-like polypeptide, said process comprising the

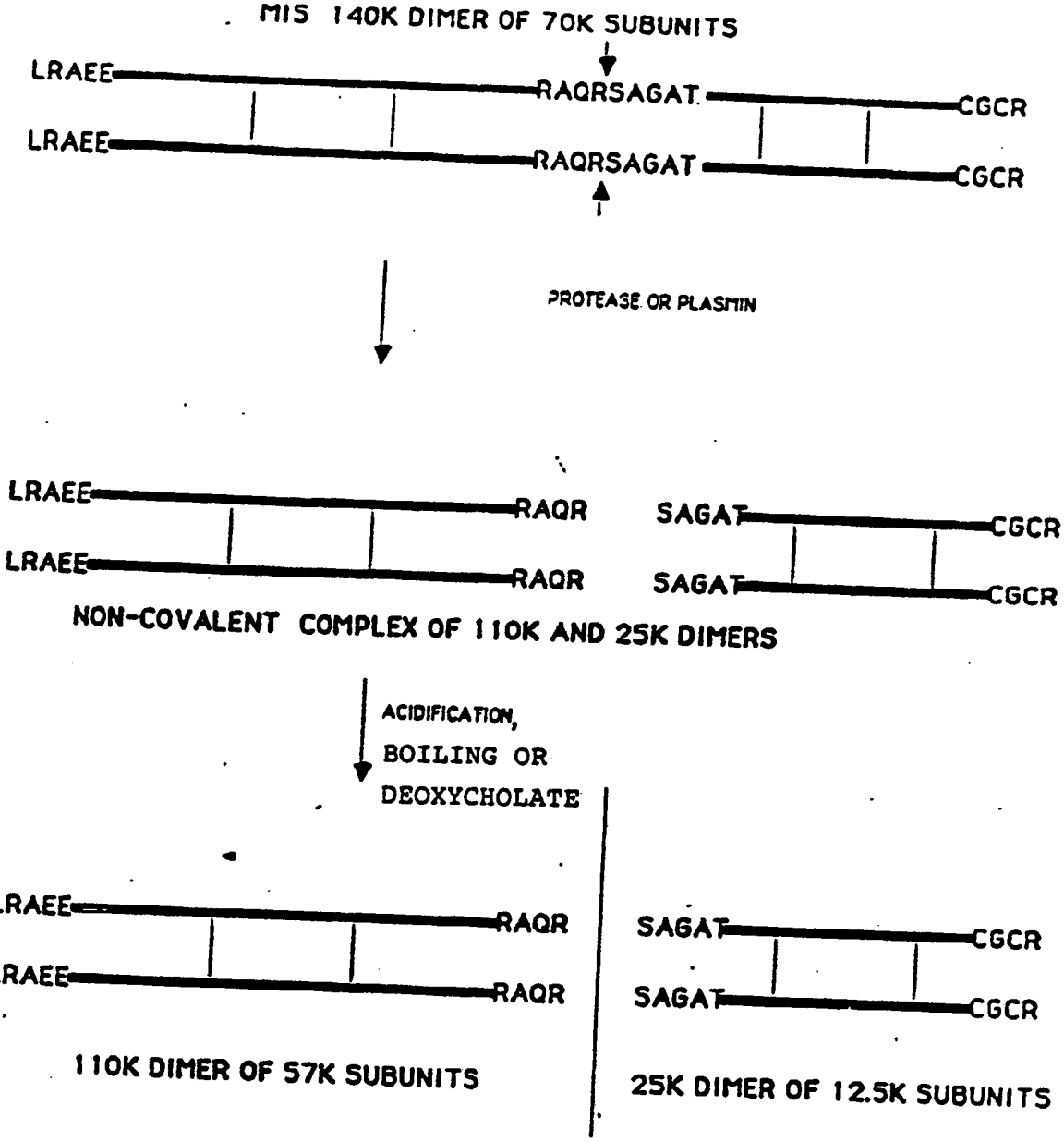
steps of treating an MIS-like polypeptide with a protease.

17. The process according to claim 16, wherein the protease is plasmin or trypsin.

18. The process according to claim 16, further comprising the step of dissociating the produced C-terminal and N-terminal dimers by boiling or acidification or treatment with deoxycholate.

19. A process for producing at least one of an N-terminal dimer or C-terminal dimer of an MIS-like polypeptide, said process comprising the step of culturing a host transformed with a DNA sequence coding for the monomer of said dimer.

FIGURE 1


MIS 140K DIMER OF 70K SUBUNITS
LRAEE
RAQRSAGAT
CGCR
LRAEE
RAQRSAGAT
CGCR
PROTEASE OR PLASMIN
LRAEE
RAQR
SAGAT
CGCR
LRAEE
RAQR
SAGAT
CGCR
NON-COVALENT COMPLEX OF 110K AND 25K DIMERS
ACIDIFICATION,
BOILING OR
DEOXYCHOLATE
LRAEE
RAQR
LRAEE
RAQR
110K DIMER OF 57K SUBUNITS
SAGAT
CGCR
SAGAT
CGCR
25K DIMER OF 12.5K SUBUNITS

2

FIGURE 2 (page 1)

DNA SEQUENCE OF HUMAN MIS GENE

```
    AAGGTCGCGGCAGAGGAGATAGGGGTCTGTCCTGCACAAACACCCCACCTTCCACTCGGCTCACTTAAGGCAGGCAGCCCAGCCCCTGGCAGCACCCACG
  1 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 100
    TTCCAGCGCCGTCTCCTCTATCCCCAGACAGGACGTGTTTGTGGGGTGGAAGGTGAGCCGAGTGAATTCCGTCCGTCGGGTCGGGGACCGTCGTGGGTGC

    ATGCGGGACCTGCCTCTCACCAGCCTGGCCCTAGTGCTGTCTGCCCTGGGGGCTCTGCTGGGGACTGAGGCCCTCAGAGCAGAGGAGCCAGCTGTGGGCA
101 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 200
    TACGCCCTGGACGGAGAGTGGTCGGACCGGGATCACGACAGACGGGACCCCCGAGACGACCCCTGACTCCGGGAGTCTCGTCTCCTCGGTCGACACCCGT
                                                                      [mature sequence
    M  R  D  L  P  L  T  S  L  A  L  V  L  S  A  L  G  A  L  L  G  T  E  A [L  R  A  E  E  P  A  V  G  T

    CCAGTGGCCTCATCTTCCGAGAAGACTTGGACTGGCCTCCAGGCATCCCACAAGAGCCTCTGTGCCTGGTGGCACTGGGCGGGGACAGCAATGGCAGCAG
201 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 300
    GGTCACCGGAGTAGAAGGCTCTTCTGAACCTGACCGGAGGTCCGTAGGGTGTTCTCGGAGACACGGACCACCGTGACCCGCCCCTGTCGTTACCGTCGTC

     S  G  L  I  F  R  E  D  L  D  W  P  P  G  I  P  Q  E  P  L  C  L  V  A  L  G  G  D  S  N  G  S  S

    CTCCCCCCTGCGGGTGGTGGGGGCTCTAAGCGCCTATGAGCAGGCCTTCCTGGGGGCCGTGCAGAGGGCCCGCTGGGGCCCCCGAGACCTGGCCACCTTC
301 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 400
    GAGGGGGGACGCCCACCACCCCCGAGATTCGCGGATACTCGTCCGGAAGGACCCCCGGCACGTCTCCCGGGCGACCCCGGGGGCTCTGGACCGGTGGAAG

     S  P  L  R  V  V  G  A  L  S  A  Y  E  Q  A  F  L  G  A  V  Q  R  A  R  W  G  P  R  D  L  A  T  F

    GGGGTCTGCAACACCGGTGACAGGCAGGCTGCCTTGCCCTCTCTACGGCGGCTGGGGGCCTGGCTGCGGGACCCTGGGGGGCAGCGCCTGGTGGTCCTAC
401 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 500
    CCCCAGACGTTGTGGCCACTGTCCGTCCGACGGAACGGGAGAGATGCCGCCGACCCCCGGACCGACGCCCTGGGACCCCCCGTCGCGGACCACCAGGATG

     G  V  C  N  T  G  D  R  Q  A  A  L  P  S  L  R  R  L  G  A  W  L  R  D  P  G  G  Q  R  L  V  V  L  H

    ACCTGGAGGAAGGTATGTGGGGCCCAGCCCCAAGCTTGGCACCGCCGTCTTCCTTCAGGTGGGCCGGGTCCTCCTAGGGAAGATCAGGGGCTGGCAGAGC
501 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 600
    TGGACCTCCTTCCATACACCCCGGGTCGGGGTTCGAACCGTGGCGGCAGAAGGAAGTCCACCCGGCCCAGGAGGATCCCTTCTAGTCCCCGACCGTCTCG

     L  E  E

    CCCCACCCTGGGCAGGGAGGCTGTGGTCTTGTTCCTAGGACTGGGTTGCGGGTCCGTGGCCTGGAAGGTGGGCACCACACTCTGTCCTGTCCCCGAAGCC
601 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 700
    GGGGTGGGACCCGTCCCTCCGACACCAGAACAAGGATCCTGACCCAACGCCCAGGCACCGGACCTTCCACCCGTGGTGTGAGACAGGACAGGGGCTTCGG

    CAGCTCTTAGACTTGCCCCTGCCTCGGTGCCAGGGAGAGAGCTGCTGCCTTCTCCCCACCCCTGAAGACGACGCAGGGCTCGGGGCCAGTGGAACCCTTC
701 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 800
    GTCGAGAATCTGAACGGGGACGGAGCCACGGTCCCTCTCTCGACGACGGAAGAGGGGTGGGGACTTCTGCTGCGTCCCGAGCCCCGGTCACCTTGGGAAG
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FIGURE 2 (page 2)

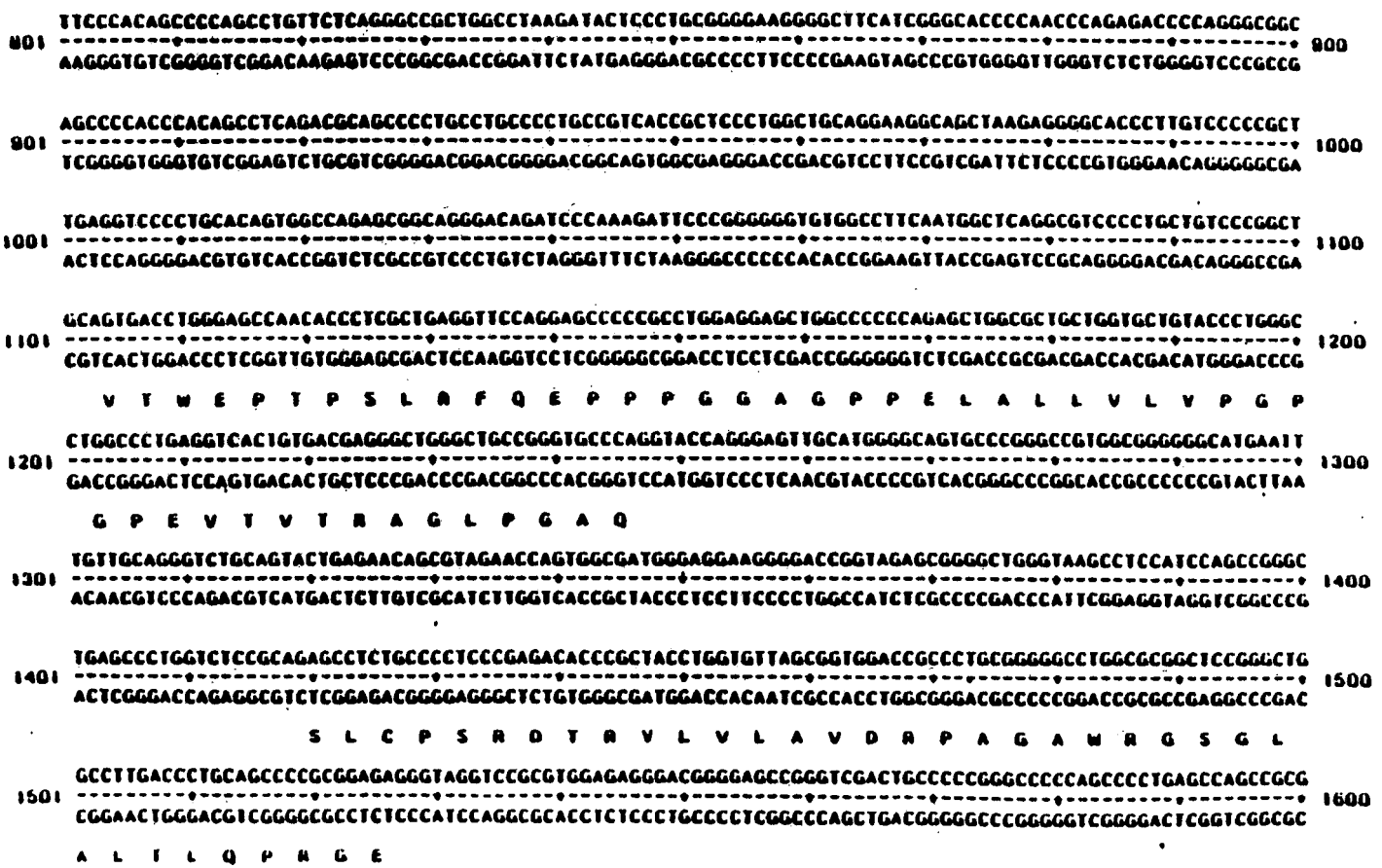


FIGURE 2 (page 3)

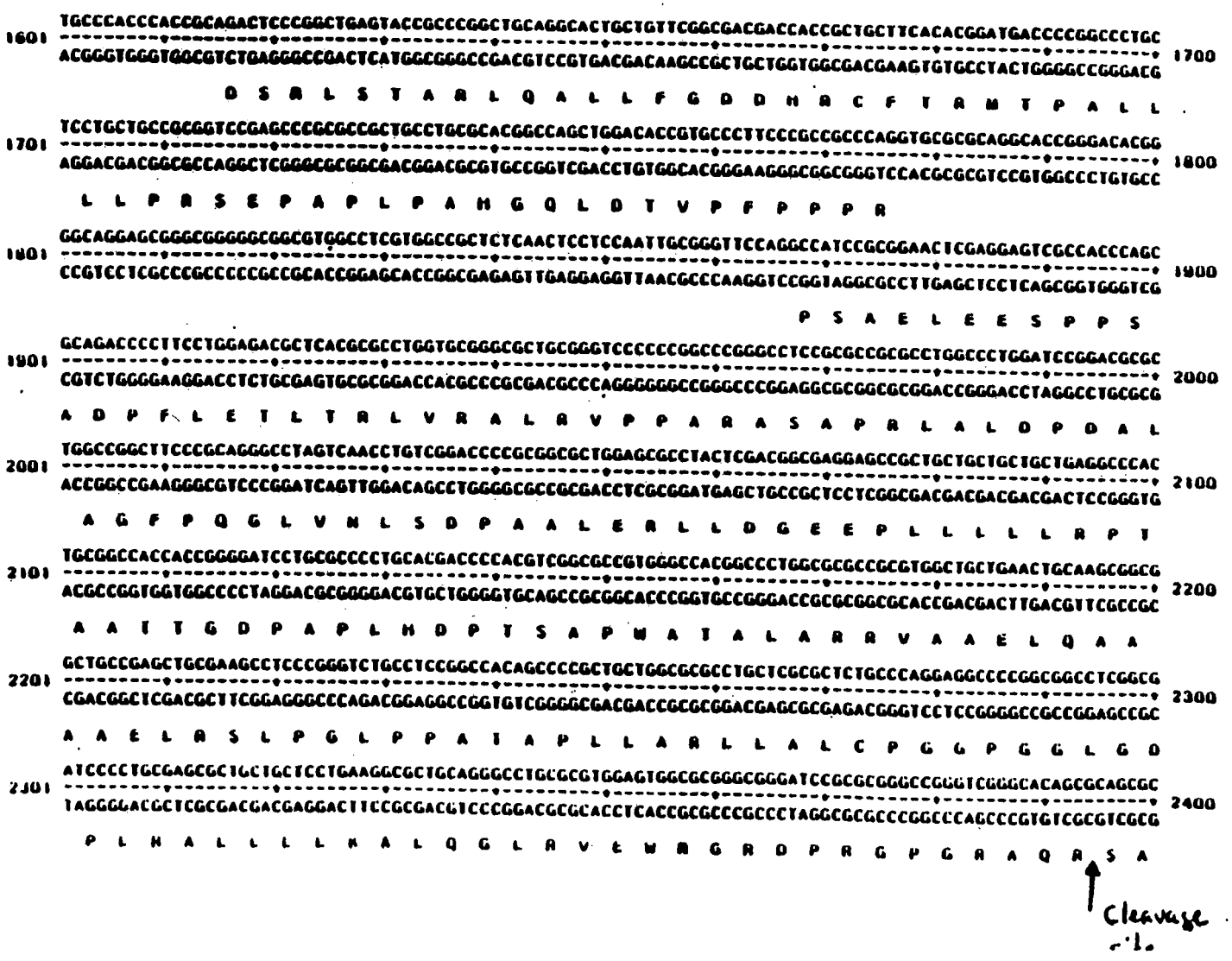


FIGURE 2 (page 4)

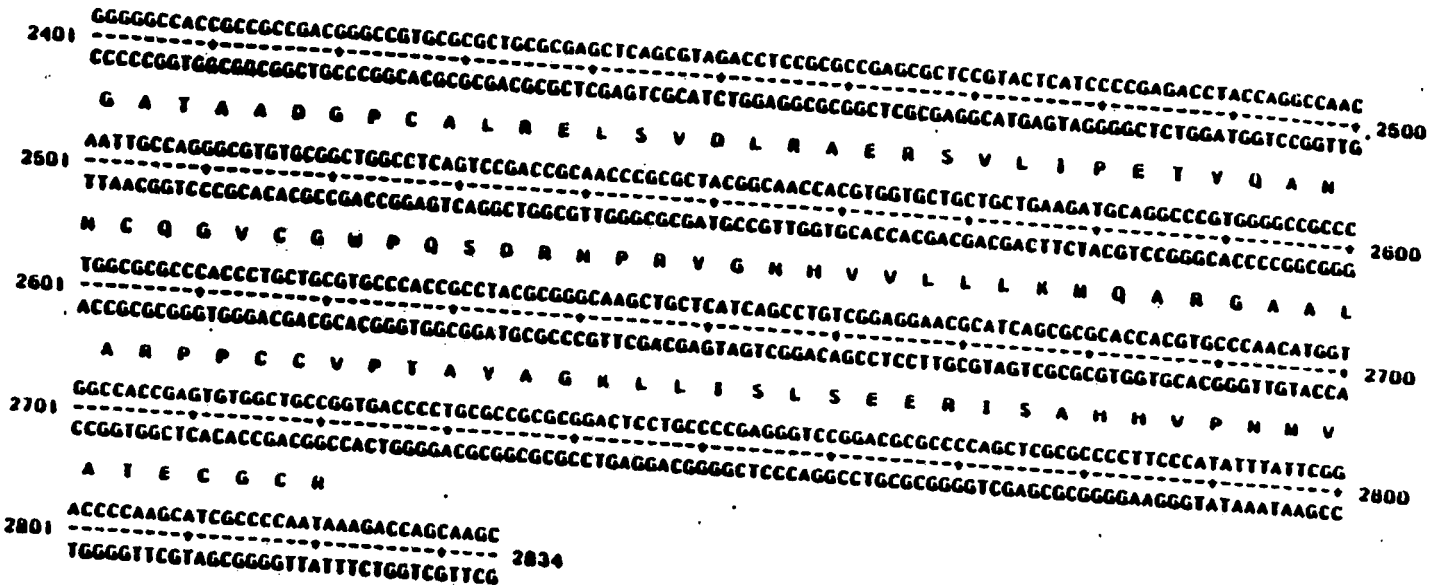


FIGURE 3 (page 1)

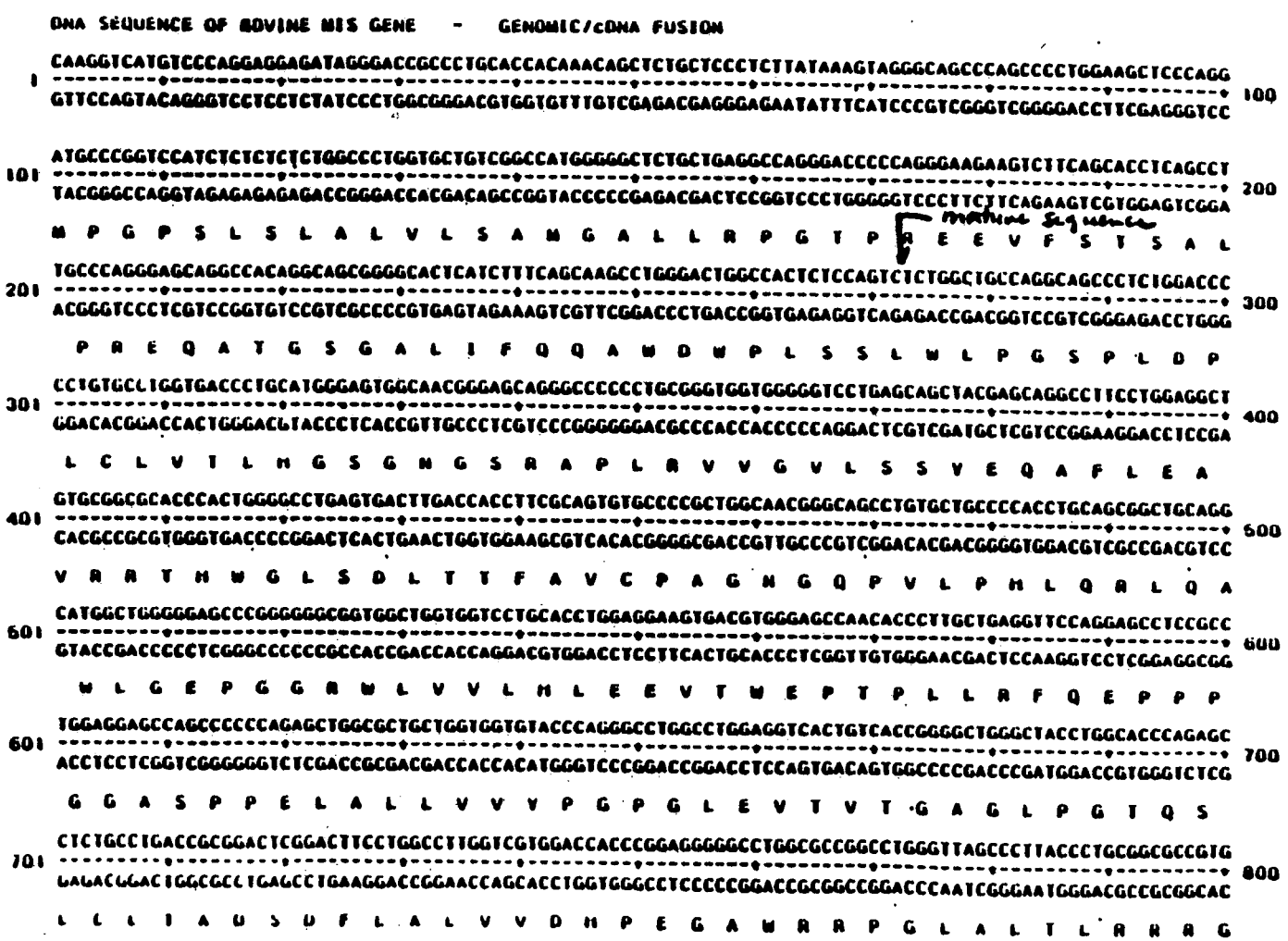
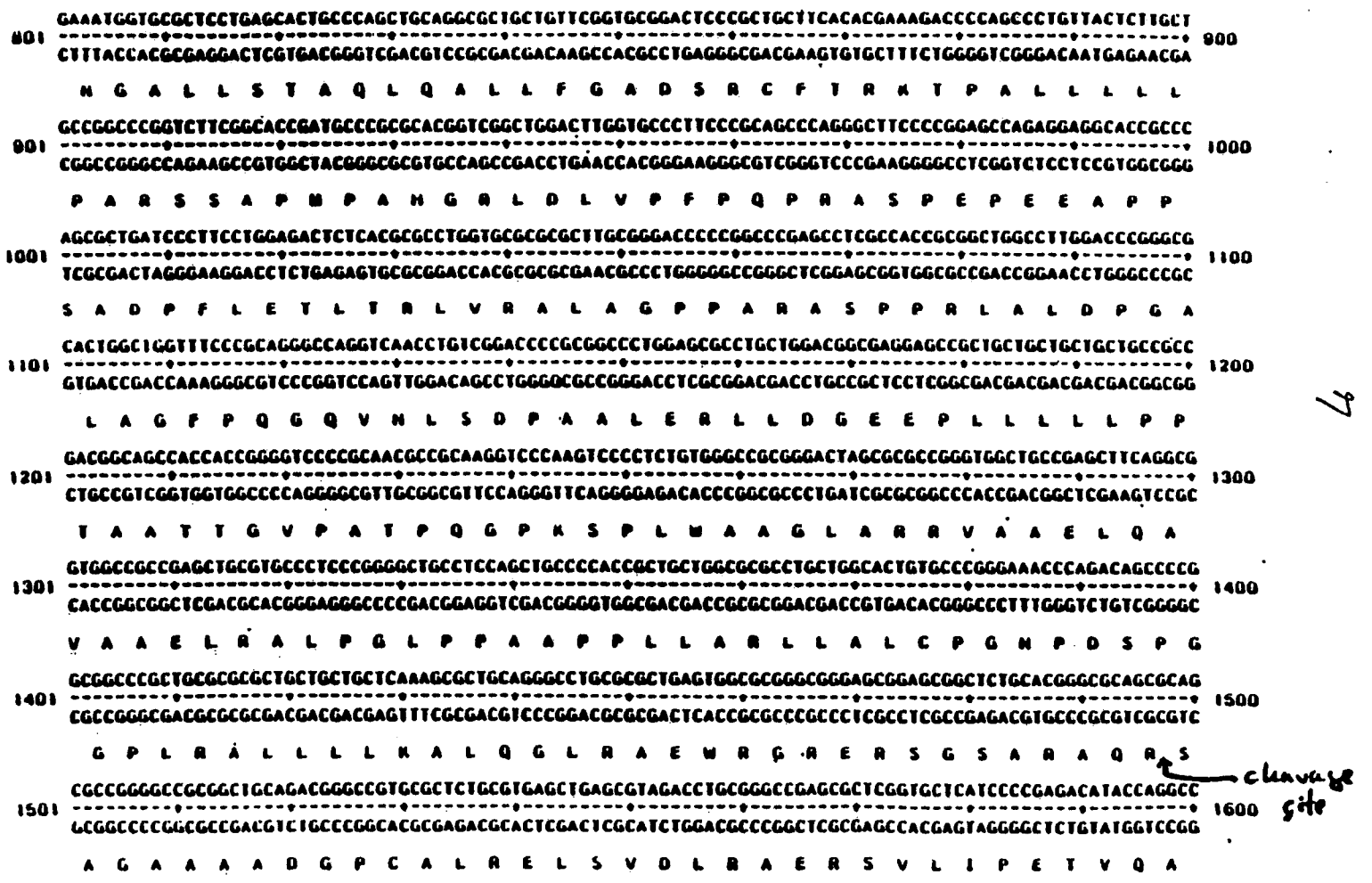


FIGURE 3 (page 2)



8

FIGURE 8 (page 3)

```
     AACAACTGCCAGGGGGCCTGCGGCTGGCCTCAGTCGGACCGCAACCCGCGCTACGGCAACCACGTGGTGCTGCTGCTAAAGATGCAGGCCCGCGGCGCCA
1601 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 1700
     TTGTTGACGGTCCCCCGGACGCCGACCGGAGTCAGCCTGGCGTTGGGCGCGATGCCGTTGGTGCACCACGACGACGATTTCTACGTCCGGGCGCCGCGGT

     N  N  C  Q  G  A  C  G  W  P  Q  S  D  R  N  P  R  V  G  N  H  V  V  L  L  L  K  M  Q  A  R  G  A  T

     CCCTGGCGCGCCCGCCCTGCTGTGTGCCCACAGCCTACACCGGCAAGCTCCTCATCAGCCTGTCCGAGGAGCGCATCAGTGCGCACCACGTCCCAAACAT
1701 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 1800
     GGGACCGCGCGGGCGGGACGACACACGGGTGTCGGATGTGGCCGTTCGAGGAGTAGTCGGACAGGCTCCTCGCGTAGTCACGCGTGGTGCAGGGTTTGTA

      L  A  R  P  P  C  C  V  P  T  A  Y  T  G  K  L  L  I  S  L  S  E  E  R  I  S  A  H  H  V  P  N  M

     GGTGGCCACCGAATGCGGCTGCCGGTGACCTCGCGCCGTGCTCCTCGTGCTGCCCCGGCCCGTATTTATTCGGACCCCGTCATTGCCCCATTAAACACGG
1801 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+ 1900
     CCACCGGTGGCTTACGCCGACGGCCACTGGAGCGCGGCACGAGGAGCACGACGGGGCCGGGCATAAATAAGCCTGGGGCAGTAACGGGGTAATTTGTGCC

     V  A  T  E  C  G  C  R

     GAAGGC
1901 ------ 1906
     CTTCCG
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Figure 4

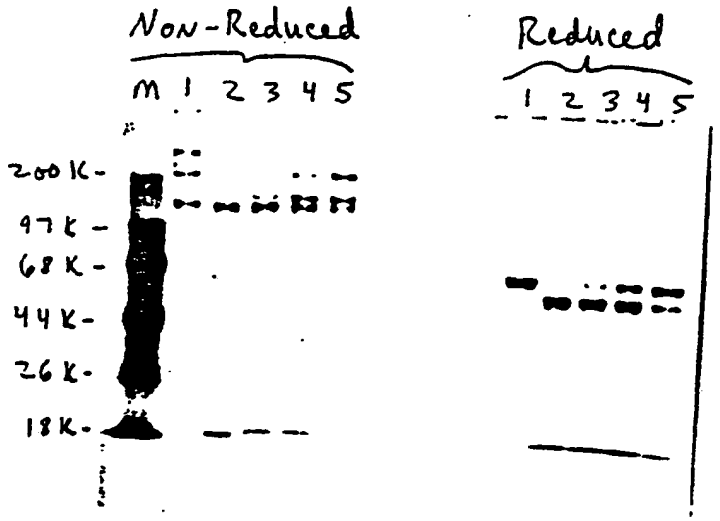


Figure 5 10/20

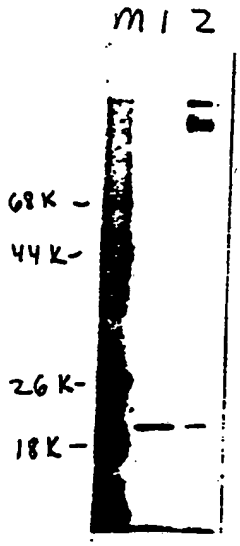


FIGURE 6

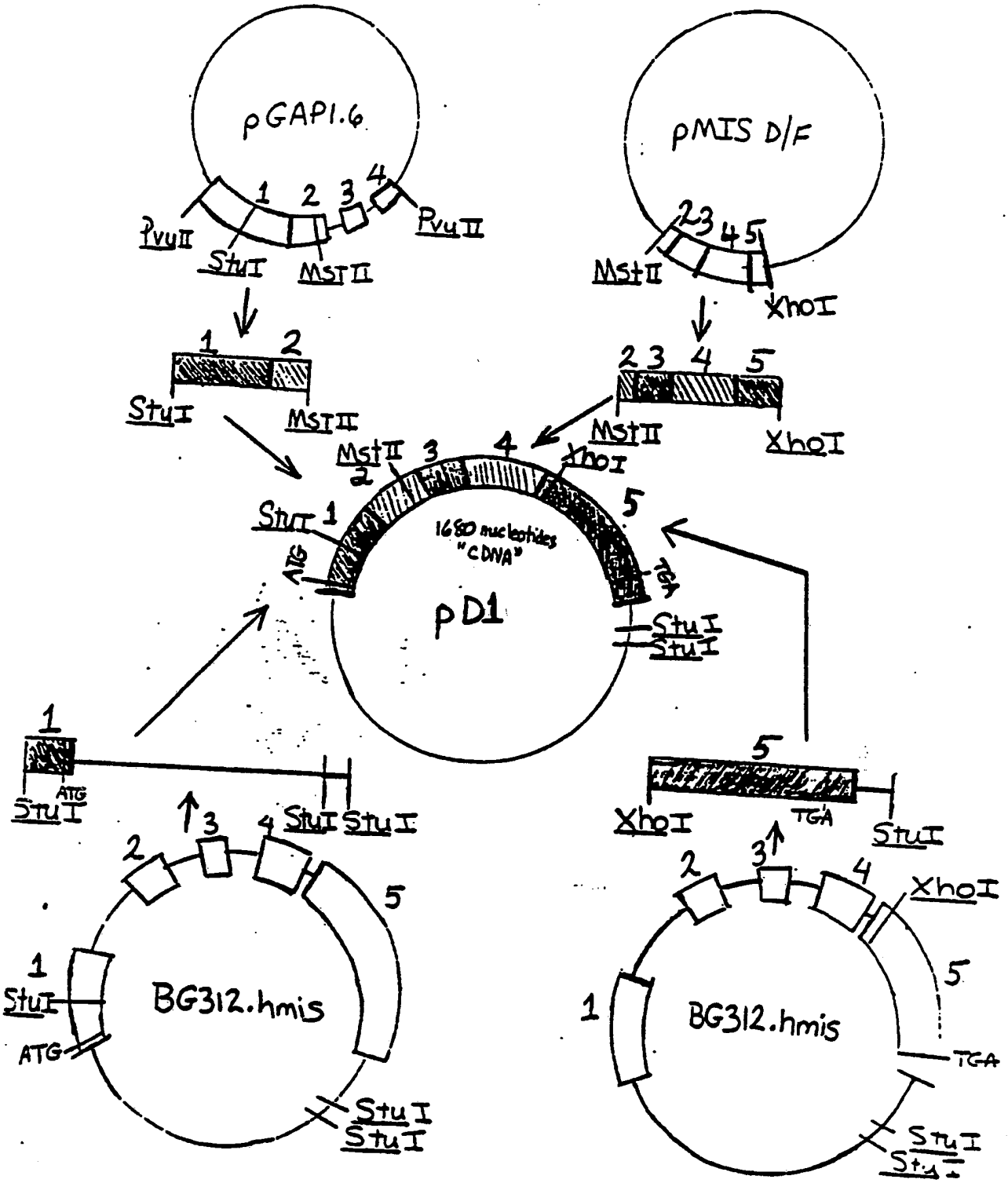
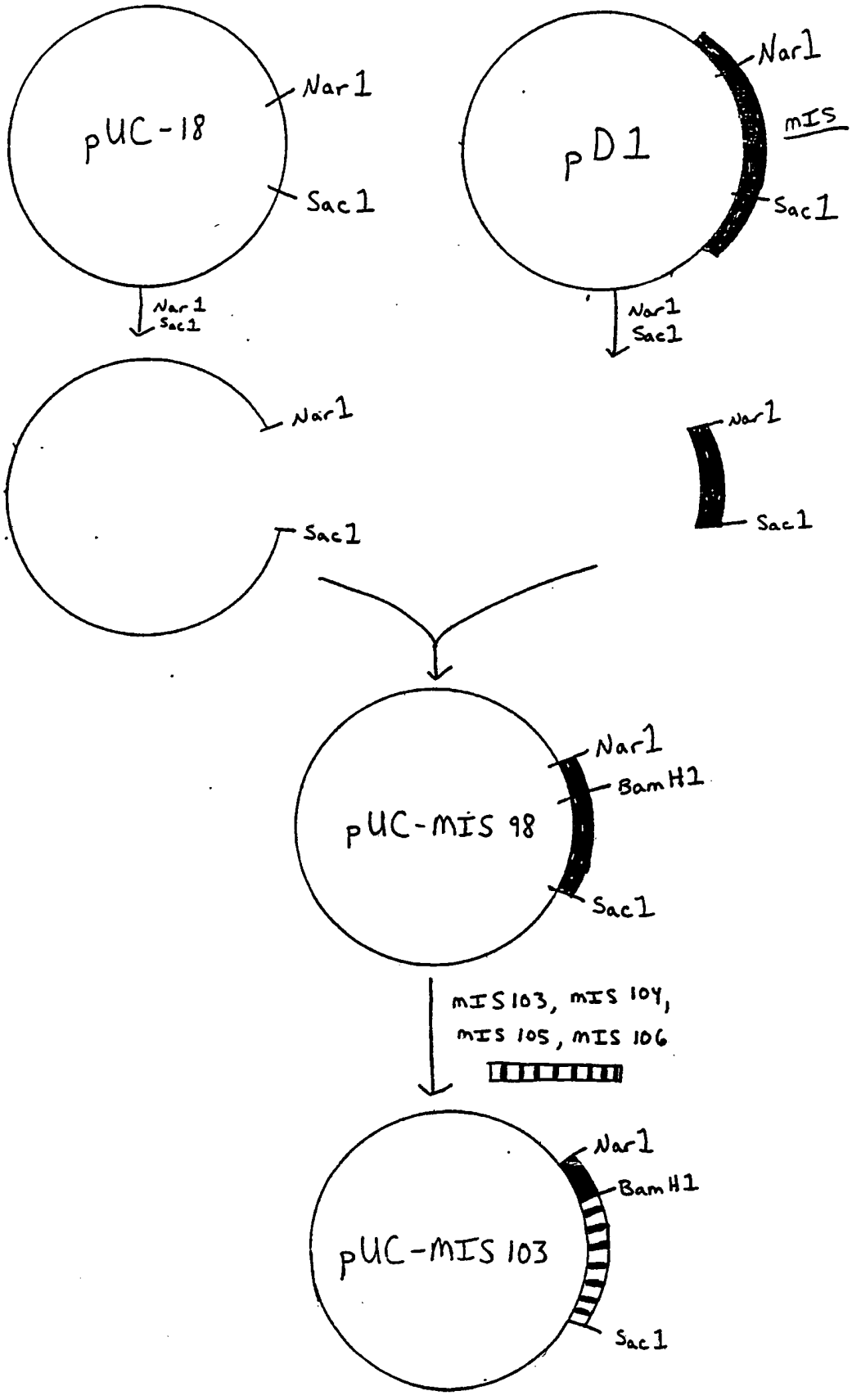

pGAP1.6
1 2 3 4
PvuII
StuI
MstII
PvuII
pMIS D/F
2 3 4 5
MstII
XhoI
1 2
StuI
MstII
2 3 4 5
MstII
XhoI
MstII 2 3 4
XhoI
StuI 1
5
1680 nucleotides "cDNA"
ATG
TGA
pD1
StuI
StuI
1
StuI
ATG
StuI
StuI
2 3 4
5
1
StuI
ATG
BG312.hmis
StuI
StuI
5
XhoI
TGA
StuI
2 3 4
XhoI
1
BG312.hmis
5
TGA
StuI
StuI

FIGURE 7A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Nar1
Sac1
Nar1
Sac1
pD1
Nar1
mIS
Sac1
Nar1
Sac1
Nar1
Sac1
Nar1
Sac1
pUC-mIS 98
Nar1
BamH1
Sac1
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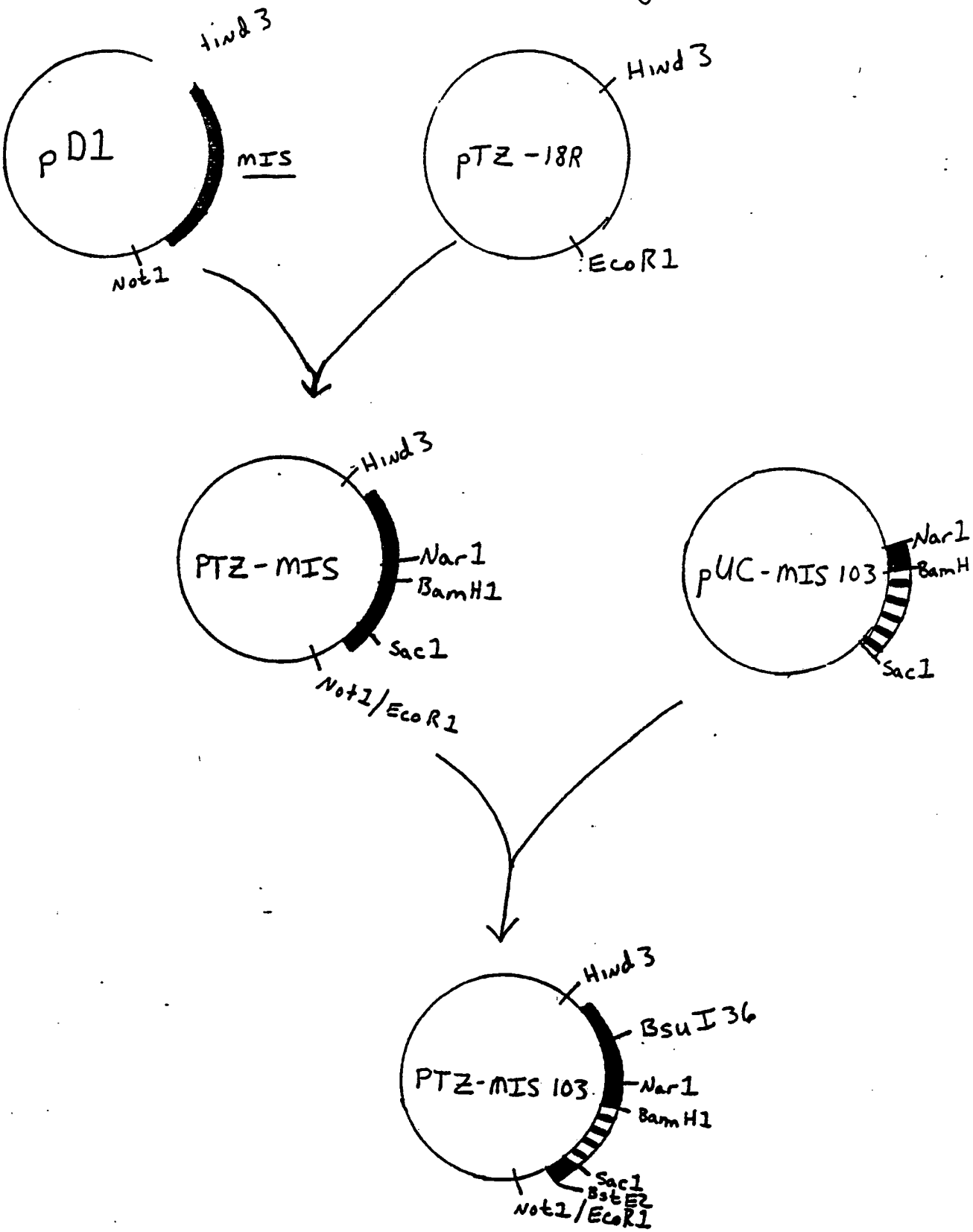
mIS 105, mIS 106
pUC-mIS 103
Nar1
BamH1
Sac1

Figure 7B


Hind 3
pD1
MIS
Not1
pTZ-18R
Hind 3
EcoR1
Hind 3
PTZ-MIS
Nar1
BamH1
Sac1
Not1/EcoR1
pUC-MIS 103
Nar1
BamH
Sac1
Hind 3
PTZ-MIS 103
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Nar1
BamH1
Sac1
BstE2
Not1/EcoR1

Figure 1C

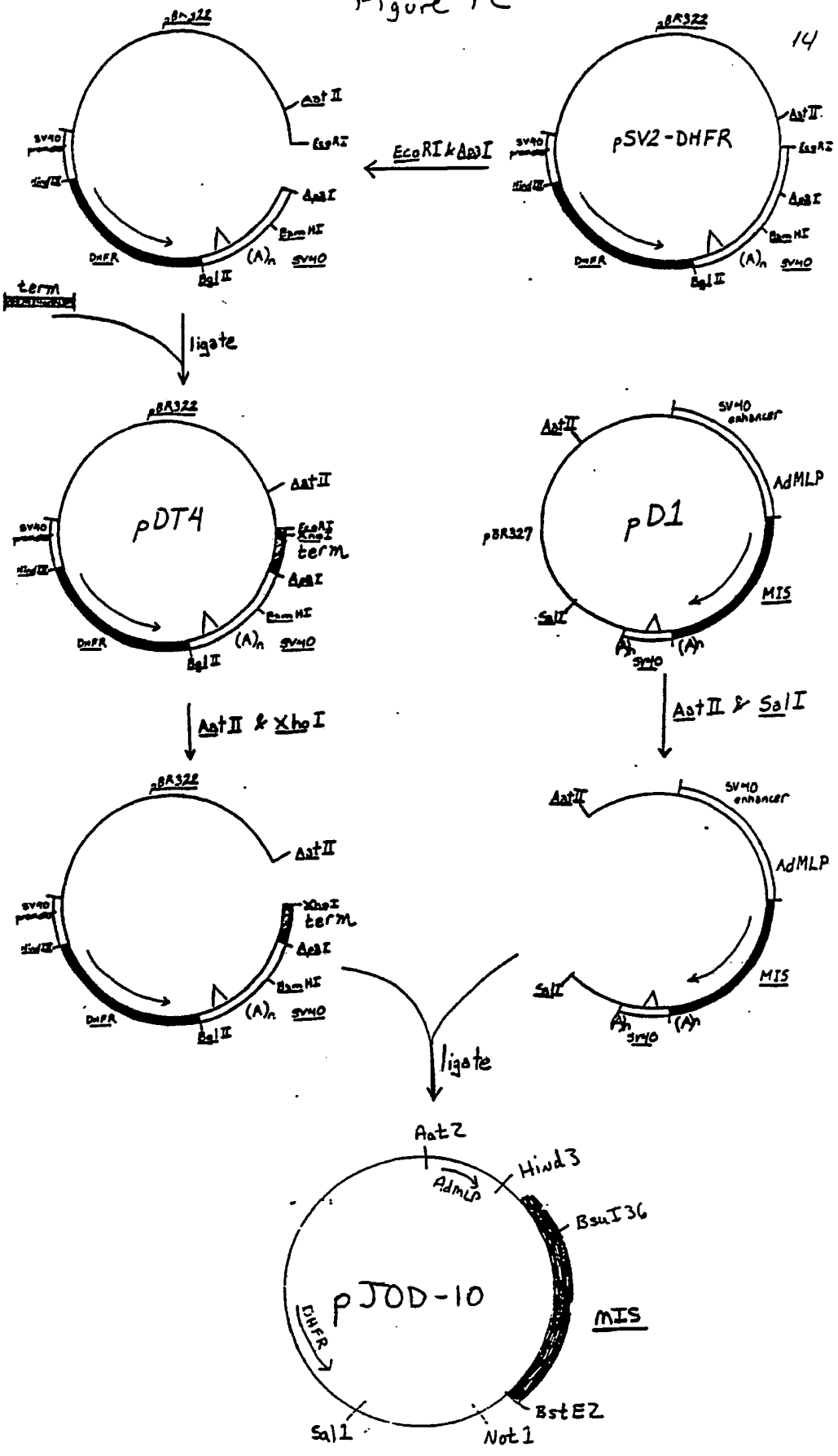

pBR322
AatII
EcoRI
SV40
HindIII
ApaI
BamHI
DHFR
(A)n
SV40
BglII
EcoRI & ApaI
pSV2-DHFR
term
ligate
pDT4
XhoI
pD1
SV40 enhancer
AdMLP
pBR327
MIS
SalI
AatII & XhoI
AatII & SalI
pJOD-10
Aat2
Hind3
BsuI36
BstE2
Sal1
Not1

Figure 7D

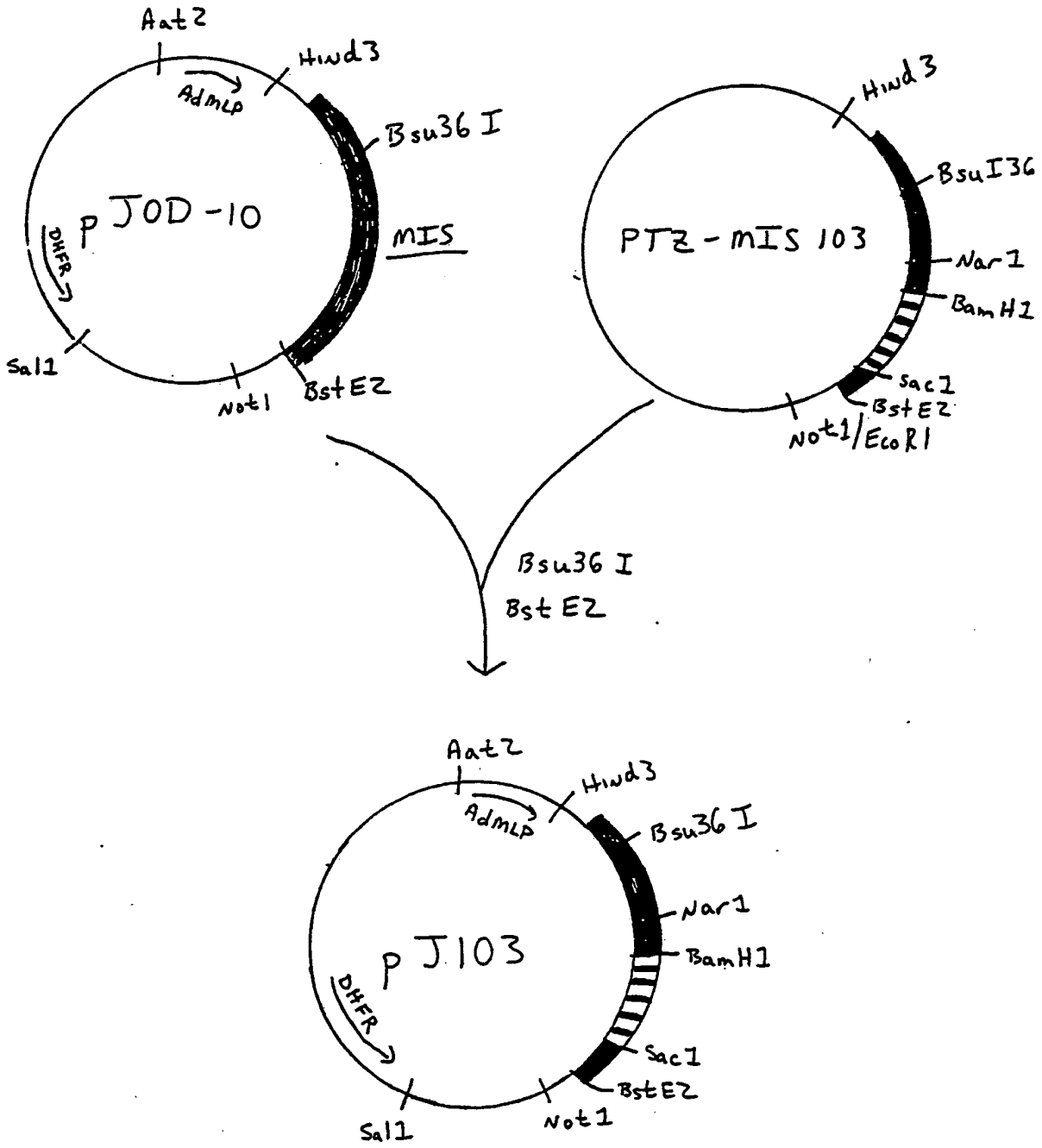

Aat2
Hind3
AdMLP
Bsu36 I
pJOD-10
MIS
DHFR
Sal1
Not1
BstE2
PTZ-MIS 103
Hind3
BsuI36
Nar1
BamH1
Sac1
BstE2
Not1/EcoR1
Bsu36 I
BstE2
Aat2
Hind3
AdMLP
Bsu36 I
Nar1
pJ103
BamH1
DHFR
Sac1
BstE2
Sal1
Not1

Figure 8

Sequence of MIS100 probe
CGGTGGCCCC CGGCTAGCGC TGTGCCCGAC

Sequence of MIS103 probe
GATCCTCGAG GACCAGGTCG TGCTCAGACG TCTGCTGGGG

Sequence of MIS104 probe
CCACTGCGGC CGATGGTCCA TGTGCACTGC GTGAGCT

Sequence of MIS105 probe
CAGTGGCCCC AGCAGACGTC TGAGCACGAC CTGGTCCTCG AG

Sequence of MIS106 probe
CACGCAGTGC ACATGGACCA TCGGCCG

Figure 9

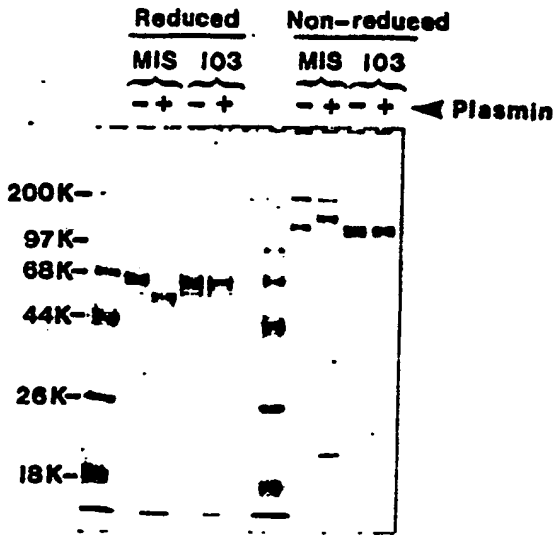

Reduced
Non-reduced
MIS 103
MIS 103
- + - +
- + - +
Plasmin
200K-
97K-
68K-
44K-
26K-
18K-

Figure 10

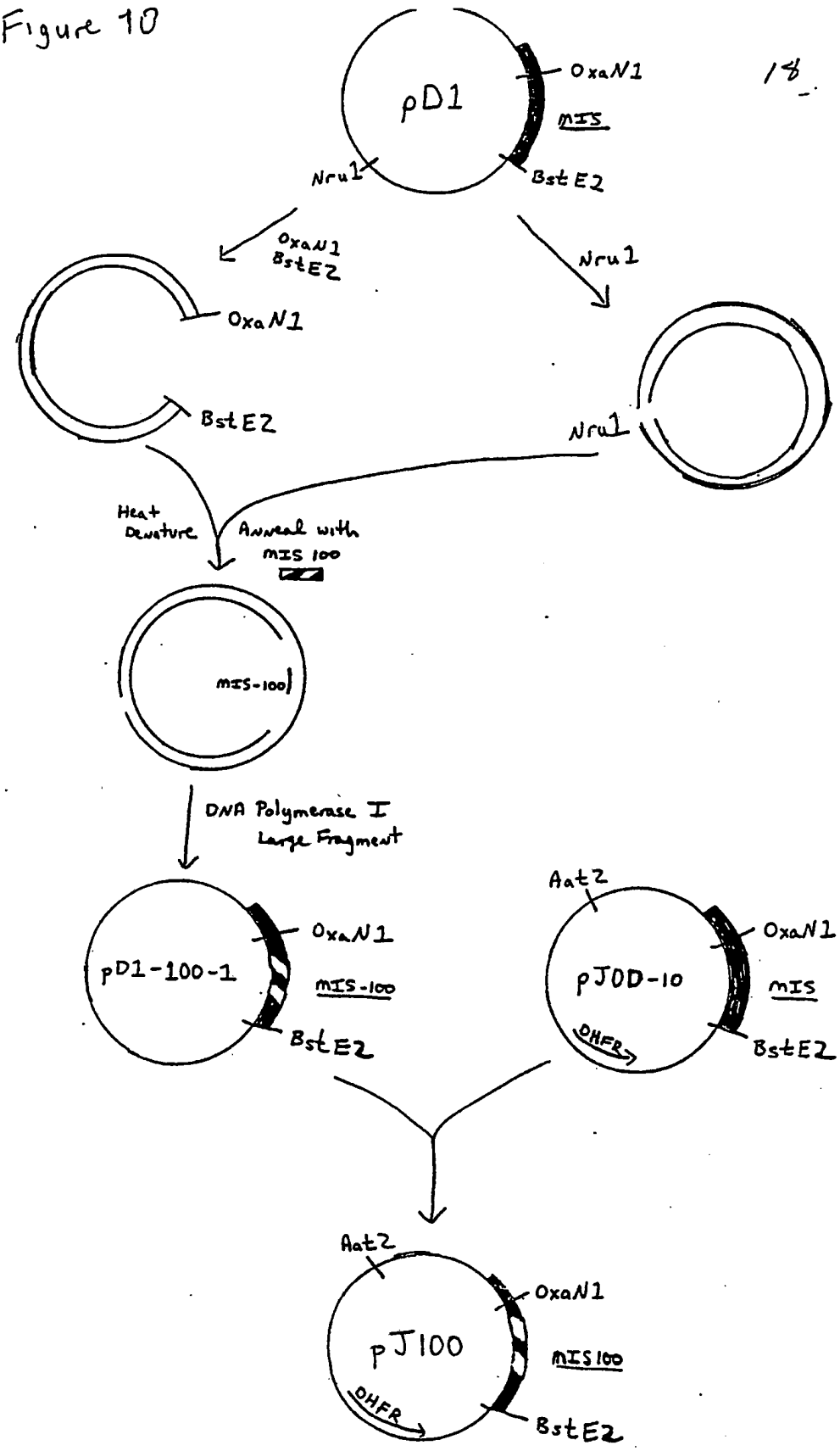

pD1
OxaN1
mIS
Nru1
BstE2
OxaN1
BstE2
OxaN1
BstE2
Nru1
Nru1
Heat Denature
Anneal with mIS 100
mIS-100
DNA Polymerase I Large Fragment
pD1-100-1
OxaN1
mIS-100
BstE2
Aat2
pJOD-10
OxaN1
mIS
DHFR
BstE2
Aat2
pJ100
OxaN1
mIS100
DHFR
BstE2

Figure 11

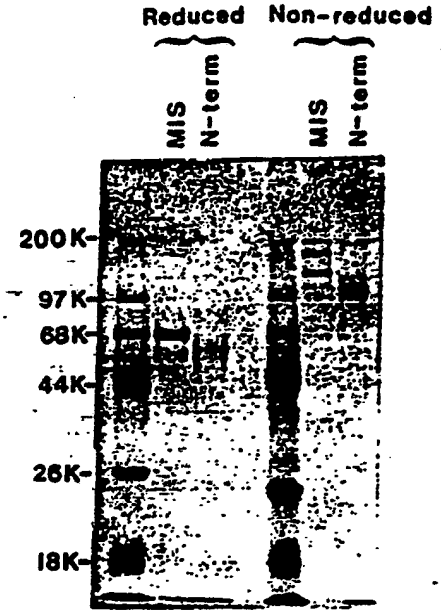
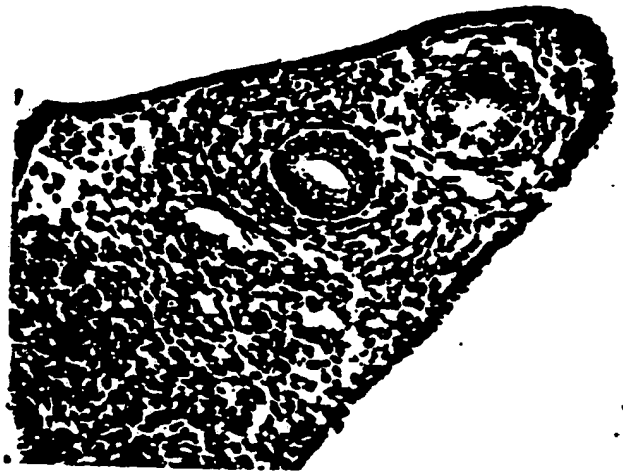

Reduced
Non-reduced
MIS
N-term
MIS
N-term
200K
97K
68K
44K
26K
18K

Figure 12

N + C
Grade 4

N
Grade 0-1



Buffer
Grade 0-1

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 89/00239

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12P 21/00; C12P 21/06; A61K 35/48; A61K 37/02; A61K 35/48; A61K 37/24

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	435/68,69,70,71,91,172.1,172.3,252.3 -252.35,320; 514/2,8,12;530/300,350,397,399;424/105;935/13

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

CA File 1967-1989
Biosis File 1967-1989

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US,A, 4,510,131 (DONAHOE ET AL) 09 April 1985. See the entire document.	9-11
Y,P	US,A, 4,753,794 (DONAHOE ET AL) 28 June 1988. See the entire document.	13-15
Y	WO,A, 00054 (THE GENERAL HOSPITAL CORPORATION) 14 January 1988. See the entire document.	13-15
Y	R.L. CATE ET AL, "Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells", Cell, Volume 45, pages 685-698, published June 1986 by MIT Press (Cambridge, Mass., USA). See the entire document.	1-11 and 13-19

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 April 1989	06 JUN 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	James Martinell

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	K.C. ROBBINS ET AL, "Human plasminogen and plasmin", Methods in Enzymology, Volume 19, pages 184-199, published 1970 by Academic Press (New York, N.Y, USA). See the entire document.	1-11 and 13-19
Y	E.L. SMITH, "Evolution of enzymes", in The Enzymes, (P.D. Boyer, ed.), published 1970 by Academic Press (New York, N.Y., USA), pages 303-307.	1-11 and 13-19

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☒ Claim numbers 12, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.